Universitat de les
Illes Balears

DOCTORAL THESIS

2015

ACCLIMATION OF PHOTOSYNTHESIS TO WATER DEFICIT AND HIGH TEMPERATURE: PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS

Juan Alejandro Perdomo López

Universitat de les
Illes Balears

DOCTORAL THESIS

2015

Doctoral Programme of Biology of the Plants

ACCLIMATION OF PHOTOSYNTHESIS TO WATER DEFICIT AND HIGH TEMPERATURE: PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS

Juan Alejandro Perdomo López

Thesis Supervisor: Dr. Jeroni Galmés Galmés

Doctor by the Universitat de les Illes Balears

Yo,

Dr. Jeroni Galmés Galmés, Profesor Titular del Departamento de Biología de la Facultat de Ciències de la Universitat de les Illes Balears

CERTIFICO:

Que el presente trabajo titulado "*Acclimation of photosynthesis to water deficit and high temperature: physiological and biochemical aspects"* presentado por Juan Alejandro Perdomo López para optar al TÍTULO univesitario oficial de DOCTOR por la Universitat de les Illes Balears dentro del Programa de Doctorado de Biología de las Plantas en Condiciones Mediterráneas, se ha realizado bajo mi dirección.

Revisado el presente trabajo, autorizo su presentación para que pueda ser juzgada por el tribunal correspondiente.

Palma de Mallorca, 24 de noviembre del 2014

Director Autor

Dr. Jeroni Galmés Galmés Juan Alejandro Perdomo López

A mi Madre y mi Hermana,

SYMBOLS AND ABBREVIATIONS LIST

Symbol	Meaning
ADP	adenosine diphosphate
A_G	gross CO_2 assimilation rate
A_N	net CO_2 assimilation rate
ATP	adenosine triphosphate
B_L	biochemical limitations
Bq	becquerel
B_t	total dry biomass
C_a	atmospheric CO_2 concentration
C_c	chloroplastic CO_2 concentration
C_i	sub-stomatal CO_2 concentration
CSIRO	Commonwealth Scientific and Industrial Research Organization
CT	control temperature
dA_N	daily net CO_2 assimilation rate
dA_N/dg_s	daily intrinsic water use efficiency
dg_s	daily stomatal conductance
D_L	diffusive limitations
dR	daily leaf dark respiration
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
ETR	electron transport rate
FAO	Food and Agriculture Organization of the United Nations
$F_{m'}$	maximum fluorescence in light-adapted state
F_m	maximum fluorescence in dark-adapted state
F_o	basal fluorescence of the dark adapted leaf
F_s	steady-state fluorescence signal
F_v/F_m	maximum quantum efficiency of PSII photochemistry
g_{bs}	bundle sheath
g_m	mesophyll conductance
GRC	growth response coefficient
GRC_{LAR}	growth response coefficient for the leaf area ratio
GRC_{LCB}	growth response coefficient for the leaf carbon balance

g_s	stomatal conductance
HT	high temperature
iA_N	instantaneous net CO_2 assimilation
iA_N/ig_s	instantaneous intrinsic water use efficiency
ig_s	instantaneous stomatal conductance
IPCC	Intergovernmental Panel on Climate Change
iR	instantaneous mitochondrial respiration
J_{max}	maximum photosynthetic electron transport rate
K_c	Michaelis constant for the carboxylation activity of Rubisco
k_{cat}^{c}	reaction turnover rate for carboxylation activity of Rubisco
K_o	Michaelis constant for the oxygenation activity of Rubisco
K_P	Michaelis constant of phosphoenol pyruvate carboxylase
K_{RuBP}	Michaelis constant for the RuBP
LA	leaf area
LAR	leaf area ratio
LCB	leaf carbon balance
LMA	leaf mass per area
LMR	leaf mass ratio
NCAR	the National Center for Atmospheric Research
O	O_2 mole fraction
PAR	photosynthetic active radiation
PEG	polyethylene glycol
PEP	phosphoenolpyruvate
PEPC	phosphoenolpyruvate carboxylase
PG	Phosphoglycolate
PGA	Phosphoglycerate
PPFD	photosynthetic photon flux density
PSII	photosystem II
PVPP	polyvivilpolypirrolidone
R	mitochondrial respiration
RAI	relative affectation index
Rca	Rubisco activase
R_L	non-photorespiratory CO_2 evolution in the light

RuBP	1,5-ribulosebiphosphate
RWC	relative water content
$S_{c/o}$	In vitro Rubisco specificity factor
TAI	temperature acclimation index temperature sensitivity index
TSI	temperature sensitivity index
V_{cmax}	maximum rate for the carboxylation activity of Rubisco
VPD	vapour pressure deficit
WD	water deficit
WW	well-watered
α	alpha
β	beta
ΔHa	activation energy
Γ^*	Gamma star
$\delta^{13}C$	carbon isotope discrimination

LIST OF PUBLICATIONS DERIVED FROM THIS THESIS

Perdomo JA, Conesa MÀ, Medrano H, Ribas-Carbó M, Galmés J (2014) Effects of long-term individual and combined water and temperature stress on the growth of rice, wheat and maize: relationship with morphological and physiological acclimation. Accepted on Physiologia Plantarum.

Perdomo JA, Carmo-Silva E, Hermida C, Flexas J, Galmés J (2014) Biochemical and diffusive limitations to photosynthesis in rice, wheat and maize grown at high temperature and under water deficit. In preparation.

Perdomo JA, Carmo-Silva E, Salvucci ME, Galmés J (2014) Rubisco and Rubisco activase play an important role in the biochemical limitations of photosynthesis in rice, wheat and maize under high temperature and water deficit. In preparation.

Perdomo JA, Cavanagh AP, Kubien DS, Galmés J (2014) Temperature dependence of *in vitro* Rubisco kinetics in species of *Flaveria* with different photosynthetic mechanisms. Submitted to Photosynthesis Research (minor revisions).

CONTENTS

ABSTRACT

Global demand of agricultural crops for food, animal feeding and biofuel experienced a notorious increase through the last decades as a consequence of the human population rising. On the other hand, the global climate change predictions foretell temperature increases and longer dry periods, especially in temperate latitudes, where most of the global crop production is located. Global climate change is principally distinguished by an increase in temperature and longer periods of drought, affecting the crop productivity worldwide. The present study was carried out with the three most important crops worldwide: rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.) and corn (*Zea mays* L.). Besides being the most grown crops, the three species were also chosen because of their different photosynthetic mechanisms, and the different environmental conditions to which they are adapted. Thereby, rice and wheat are C_3 species from warm and cold environments, respectively, and corn is a C_4 species from warm environments. With the aim to test for the response of the three crops to the stresses due to the expected climate change, i.e., high temperature and drought, plants were grown at 38ºC and 25ºC (control) and, within each temperature, a batch of plants was grown under water deficit conditions (WD) and another batch was well watered (WW). In all cases, physiological, biochemical and growth-related parameters were measured in order to evaluate the effect of the imposed stresses. Especially related to physiological parameters like CO_2 assimilation and to diffusive processes, another outcome of this experimental design was also measure these parameters at 25ºC and 38ºC to determine the ability of the studied crops to adapt and acclimate to such stressing conditions. Both stresses had a negative effect on plants, but the impact was different depending on the measured parameters. High temperature had a bigger effect in biomass, biomass allocation and mitochondrial respiration, the two first, especially in wheat and corn. Instead, in the three species, photosynthetic and diffusive parameters as A_N and g_s showed a higher constraint under water deficit than at high temperature. With regard to biochemical measurements, Rubisco *in-vitro* parameters showed the same trend that A_N, whereas Rubisco activity, concentration and carbamylation state were more affected by high temperature than by water deficit. Rubisco activity tends to decrease as temperature increases because of the higher production of inhibitors. In this study the apparent rate of Rubisco activation (k_a) increased with temperature, which did not correlate with the decrease in Rubisco activity and carbamylation state. Also, some

parameters only showed a negative effect when both stresses were acting at a time. This would indicate the importance of the additive effect of temperature rising and drought stresses.

RESUMEN

La demanda mundial de cultivos agrícolas para la alimentación ha experimentado un aumento notorio a través de las últimas décadas como consecuencia de la creciente población humana. Por otra parte, las predicciones globales de cambio climático predicen aumentos de temperatura y períodos de sequía más largos, especialmente en latitudes templadas, donde se encuentra la mayor parte de la producción mundial de cultivos. El presente estudio se llevó a cabo con tres de los cultivos más importantes a nivel mundial: arroz (*Oryza sativa* L.), trigo (*Triticum aestivum* L.) y maíz (*Zea mays* L.). Además de tres de los cultivos más demandados a nivel mundial, estas tres especies también fueron escogidas debido a sus diferentes mecanismos fotosintéticos, y a las diferentes condiciones ambientales a las que están adaptadas. De esta manera, el arroz y el trigo son especies C_3 de ambientes cálidos y fríos, respectivamente, y el maíz es una especie C_4 de ambientes cálidos. Con el objetivo de evaluar la respuesta de estos tres cultivos a las tensiones derivadas del cambio climático, es decir, alta temperatura y la sequía, las plantas se cultivaron a 38ºC y 25ºC, y dentro de cada temperatura, un lote de plantas era cultivado bajo condiciones de déficit hídrico (WD) y otro, como bajo capacidad de campo (WW). En todos los casos, se midieron los parámetros de crecimiento, fisiológicos y bioquímicos con el fin de evaluar el efecto de los dos estreses impuestos. Con relación a los parámetros fisiológicos, como la asimilación de CO_2 y los procesos difusivos, estos se midieron a 25ºC y 38ºC para determinar la capacidad de estos cultivos para adaptarse y aclimatarse a la alta temperatura y el déficit hídrico. Ambas tensiones tuvieron un efecto negativo sobre las plantas, pero el impacto fue diferente dependiendo de los parámetros medidos. La alta temperatura tuvo un mayor efecto perjudicial en la producción de biomasa y la respiración mitocondrial, especialmente en trigo y maíz. En cambio, en las tres especies, la capacidad fotosintética y los parámetros de difusión como g_s mostraron una mayor restricción bajo déficit de agua que a alta temperatura. Con respecto a las mediciones bioquímicas, los parámetros de Rubisco *in vitro* mostraron la misma tendencia que A_N, mientras que la actividad Rubisco, la concentración y el estado carbamilación fueron más afectados por las altas temperaturas que por el déficit hídrico. Finalmente, algunos de los parámetros medidos en esta tesis sólo mostraron un efecto negativo cuando ambas tensiones actuaron conjuntamente. Esto indica la importancia de estudiar la interacción entre estos dos estreses.

Chapter 1

INTRODUCTION

1.1. Global climate change

According to the United Nations Intergovernmental Panel on Climate Change (IPCC 2013), increased concentration of greenhouse gases in the atmosphere is causing climate change in terms of higher temperatures, changing patterns of precipitation and water availability and increased frequency of extreme weather events such as floods and droughts. These predicted events will have adverse impacts on agriculture yields (FAO 2010).

The increase in the carbon dioxide concentration has been the principal factor causing warming over the past 50 years (IPCC 2013). Its concentration has been building up in the Earth's atmosphere since the beginning of the industrial era in the mid-1700's, primarily due to the burning of fossil fuels (coal, oil, and natural gas), and the clearing of forests (Alley, Lynch-Stieglitz & Severinghaus 1999). Human activities have also increased the emissions of other greenhouse gases, such as methane, nitrous oxide and halocarbons. These emissions are thickening the blanket of heat-trapping gases in Earth's atmosphere, causing surface temperatures to rise (Alley *et al.* 1999).

The increment of gas emissions has different consequences, among which the most direct is the increase in the average temperature of the globe. The global average surface temperature was increased by 0.6 ± 0.2°C since the late 19th century (Griggs 2001; Hansen *et al.* 2010). The average temperature around the globe in 2011 was 0.51°C warmer than the mid-20th century baseline. The first 11 years of the 21st century experienced notably higher temperatures compared to the middle and late 20th century (Hansen *et al.* 2010). By 2100 global average temperature is expected to warm at least twice as much as it has during the last 100 years. All climate projections predict an increase in average global temperature of 2°C to 5°C by 2100, depending on the level of future greenhouse gas emissions and the outcomes from various climate models (Fig. 2; from National Research Council 2010).

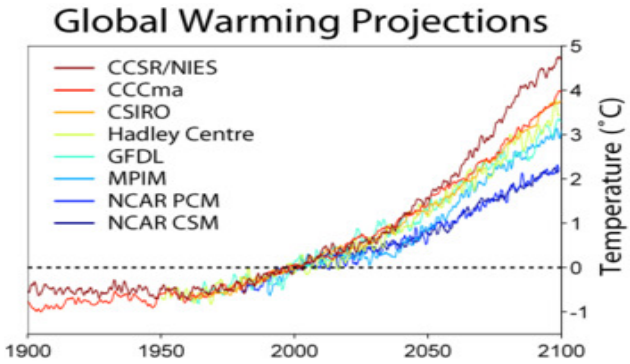

Figure 2. Predicted changes in average surface temperature according to a range of global climate models using an special report on emissions Scenarios (SRES; National Research Council 2010)

One of the consequences of increasing temperatures is the alteration of the precipitation patterns. Drought can be defined as a period of unusually dry weather that persists long enough to cause environmental problems, such as crop damage and water supply shortages (Dracup, Lee & Paulson 1980). With the temperatures rising due to global climate change, more moisture evaporates from land and water, leaving less water behind (Alley *et al.* 1999). Observations show that changes are occurring in the amount, intensity, frequency and type of precipitation (Dore 2005), and this precipitation is not distributed evenly over the globe. The average distribution is governed primarily by atmospheric circulation patterns, the availability of moisture, which are both influenced by temperature, as well as surface terrain effects (Dore 2005). Thus, anthropogenic changes in temperature are expected to alter precipitation patterns (Fig. 3). Pronounced increases in precipitation over the past 100 years have been observed in eastern North America, southern South America, and northern Europe. Decreases have been seen in the Mediterranean, most of Africa, and southern Asia. Changes in the geographical distribution of droughts and flooding have been complex. In some regions, there have been increases in the occurrences of both droughts and floods (Alley *et al.* 1999). A recent study reports a global increase of area in drought of 20-35%, depending on the climate model used (Sheffield, Wood & Roderick 2012).

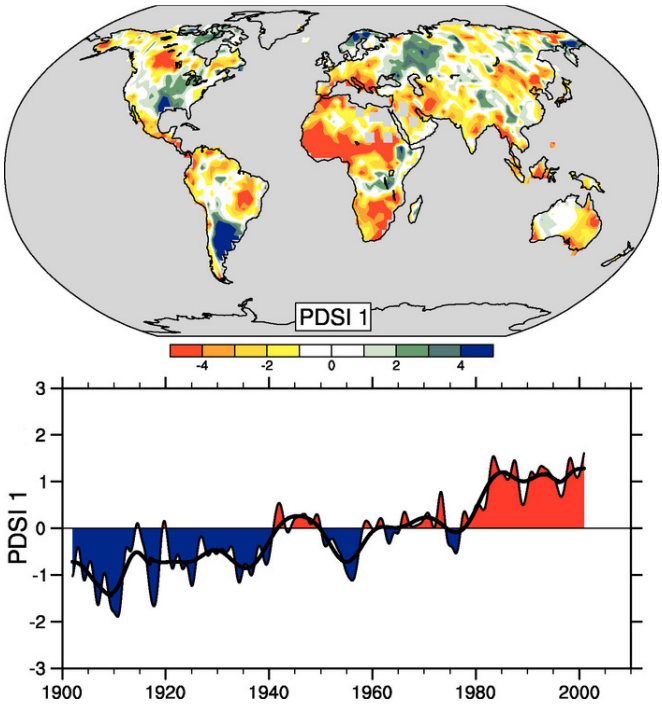

Figure 3. The most important spatial pattern (top) of the monthly Palmer Drought Severity Index (PDSI) for 1900 to 2002. The PDSI is a prominent index of drought and measures the cumulative deficit (relative to local mean conditions) in surface land moisture by incorporating previous precipitation and estimates of moisture drawn into the atmosphere (based on atmospheric temperatures) into a hydrological accounting system. The lower panel shows how the sign and strength of this pattern has changed since 1900. Red and orange areas are drier than average and blue and green areas are wetter than average when the values shown in the lower plot are positive (negative). The smooth black curve shows decadal variations. The time series approximately corresponds to a trend, and this pattern and its variations account for 67% of the linear trend of PDSI from 1900 to 2002 over the global land area (IPCC, 2007).

Because climate-change simulations are inherently uncertain, two climate models have been used to simulate future climate, using the scenario of the US National Center for Atmospheric Research (NCAR; Nelson *et al.* 2009) and the Commonwealth Scientific and Industrial Research Organization (CSIRO; Nelson *et al.* 2009). Both scenarios project higher temperatures in 2050 (Fig. 4), resulting in higher evaporation and increased precipitation as this water vapor returns to Earth (Nelson *et al.* 2009). The "wetter" NCAR scenario estimates average precipitation increases on land of about 10 percent, whereas the "drier" CSIRO scenario estimates increases of about 2 percent (Fig. 5). Figure 4 shows the change in average maximum temperature between 2000 and 2050 for the CSIRO and NCAR scenarios. Figure 5 shows changes in average precipitation. Focusing on the Mediterranean, predictions foresee increases in temperature between 2 and 6ºC, and decreases in precipitation between 75 and 200 mm

by 2050. In each set of figures, the legend colors are identical; a specific color represents the same change in temperature or precipitation across the two scenarios.

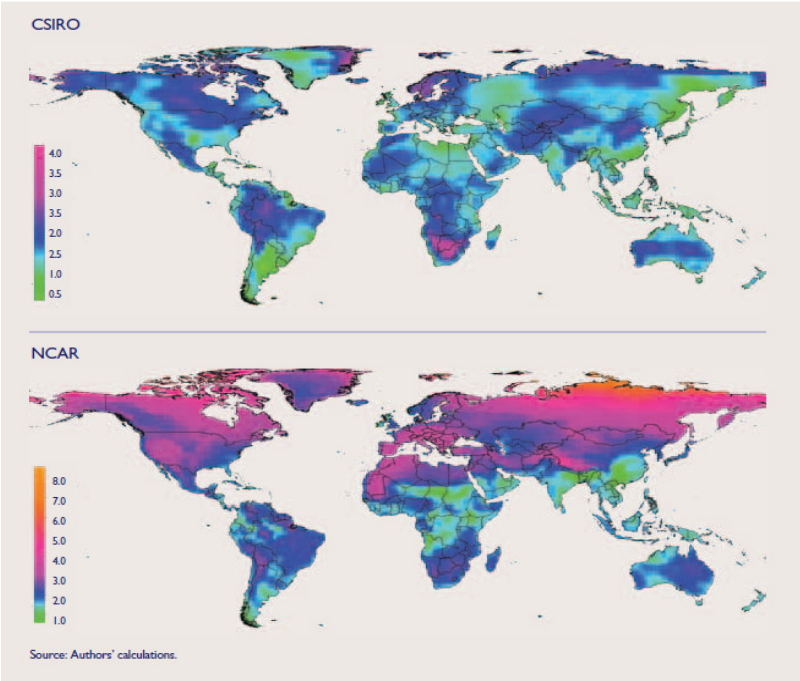

Figure 4. The change in average maximum temperature (ºC) between 2000 and 2050 for the CSIRO and NCAR scenarios (Nelson *et al.* 2009).

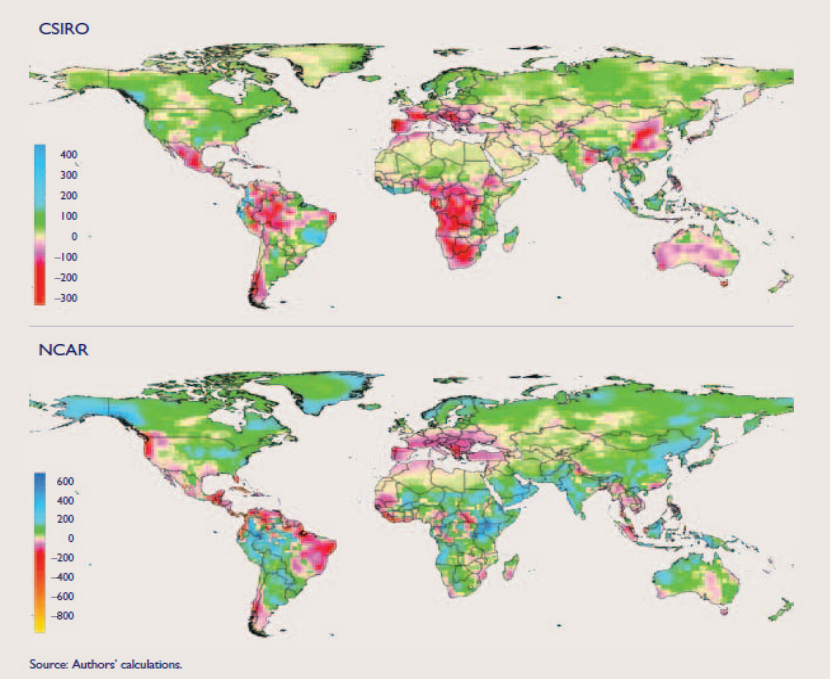

Figure 5. The change in average precipitation (mm) between 2000 and 2050 for the CSIRO and NCAR scenarios (Nelson *et al.* 2009).

1.2. Climate change repercussions in crop productivity

Agriculture is extremely vulnerable to climate change (Turral, Burke & Faurès 2011). Higher temperatures eventually reduce crop yields, while encouraging weed and pest proliferation (Nelson *et al.* 2009; Reynolds & Rodomiro 2010; Gornall *et al.* 2010). Changes in precipitation patterns increase the likelihood of short-run crop failures and long-run production declines. Although there will be crop gains in some regions of the world, the overall impacts of climate change on agriculture are expected to be negative (Nelson *et al.* 2009; Reynolds & Rodomiro 2010; Gornall *et al.* 2010). Populations in the developing world, which are already vulnerable and food insecure, are likely to be the most seriously affected. In 2005, nearly half of the economically active population in developing countries – 2.5 billion people – relied on agriculture for their livelihood. Today, 75% of the world's poor live in rural areas (Nelson *et al.* 2009). By 2050, it is predicted that there will be between 8.0 and 10.4 billion people on Earth, with a median value of 9.1 billion. If all of these people are to be fed sufficiently, total food consumption will have to increase by 50–70% (Smil 2005; FAO 2010). The report by IPCC (2013) predicted that global food prices would increase due to failure of supply to keep full pace with demand only if global mean temperature rises by more than 5.5ºC (Christensen *et al.* 2007). IPCC assumed yield improvements of 80% by 2050, continuing the trend of the second half of the 20th century. However, it is not clear if we can expect the year–on–year increases of the last half of the 20th century to continue (Long & Ort, 2010). For example, China is the world's largest producer of rice, the world's most important food in terms of the number of people dependent on it as a direct source of calories. Between 1987 and 1997 its average production (t/ha) rose 17%, but only 2% between 1997 and 2007 (FAO 2010), despite continued genetic improvement. Wheat, the second most important caloric source for humans, rose 20% in production from 1987 to 1997, but global yield declined 1% from 1997 to 2007 (Fig. 6). Of the world's three most important grains, only corn maintained the rate of increase of the 1970's and 1980's into the most recent decade (FAO 2010). In contrast to IPCC projections, this decline in crop productivity suggests that capacity for continued increase is approaching a ceiling.

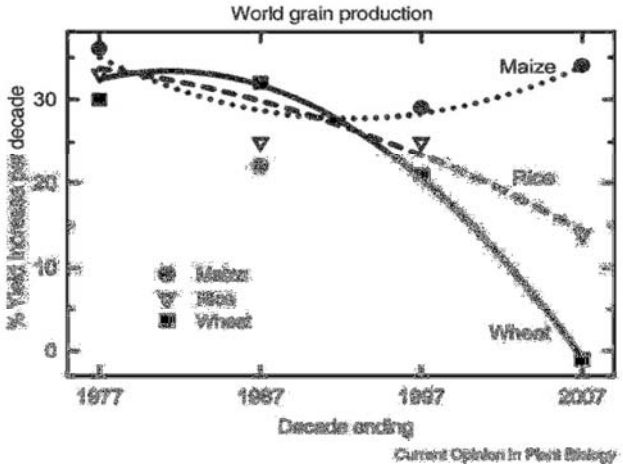

Figure 6. Increase (%) in world production per decade of the three major cereal grains, based on UN Food and Agriculture Organization records; with 2007 as the last year for which complete data were available. Global production of grain in 2007 was corn 788 Mt, paddy rice 657 Mt and wheat 611 Mt, compared to 272, 277 and 294 Mt, respectively in 1967.Wheat and rice gains have declined over the past two decades, only corn has continued to maintain the increases of earlier decades (Long & Ort, 2010)

Direct effects on crop yields due to rising temperatures and changes in rainfall patterns have been already reported (Nguyen 2008; Challinor *et al.* 2009; Long & Ort 2010). Australia, historically among the four largest wheat exporters, has in the last seven years suffered unprecedented droughts and its wheat yields averaged 25% less than the previous seven years (FAO 2010). Likewise, the crop production in Europe has also changed in response to climate change (Porter & Semenov 2005). Figure 7 suggests that inter-annual variation in wheat yields has increased since the mid–1980s, although the southern European countries have lower rates of wheat yield increase than northern ones, suggesting that climate plays a determining role in yield production (Schär *et al.* 2004).

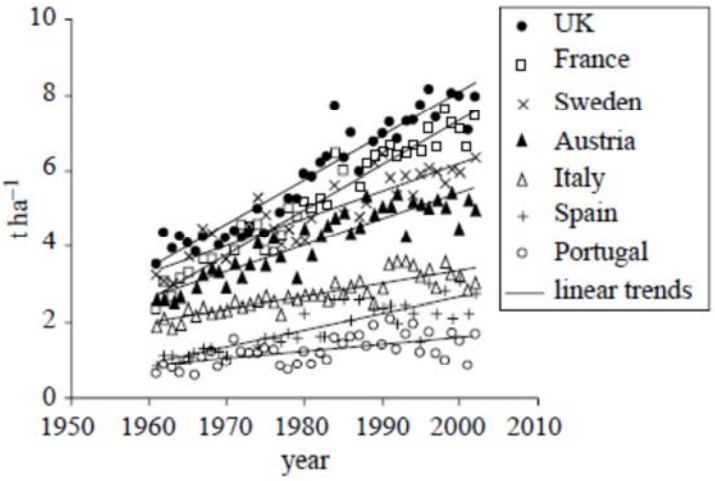

Figure 7. Observed (FAO 2003) grain yields of wheat for selected countries in Europe (Porter & Semenov 2005)

Future predictions about the effect of global climate change on crop production should consider different scenarios designed to explore future developments in the global environment. Table 1 reports the effects of climate change on crop production in 2050 compared to production without climate change, based on the NCAR and CSIRO scenarios, accounting for both the direct changes in yield and area caused by climate change and autonomous adaptation as farmers respond to changing prices with changes in crop mix and input use (Nelson *et al.* 2009). The negative effects of climate change on crop production are especially pronounced in Sub-Saharan Africa and South Asia. In South Asia, the climate scenario results in a 14% decline in rice production relative to the no–climate–change scenario, a 44 to 49% decline in wheat production, and a 9 to 19% fall in corn production. In Sub-Saharan Africa, rice, wheat, and corn yield estimated declines with climate change are 15, 34 and 10%, respectively. For East Asia and the Pacific, the results are mixed and depend on both the crop and the model used. Rice production declines by around 10%, wheat production increases slightly, and corn production declines with the drier CSIRO scenario but increases with the NCAR scenario. Comparing average production changes, developing countries fare worse for all crops under both the CSIRO and NCAR scenarios than do developed countries (Nelson *et al.* 2009).

Table 1. Climate-change effects on crop production. The rows labeled "2050 No CC (% change)" indicate the percent change between production in 2000 and 2050 with no climate change. The rows labeled "CSIRO (% change)" and "NCAR (% change)" indicate the additional percent change in production in 2050 due to climate change relative to 2050 with no climate change (Nelson *et al.* 2009).

Agricultural product	South Asia	East Asia and the Pacific	Europe and Central Asia	Latin America and the Caribbean	Middle East and North Africa	Sub-Saharan Africa	Developed countries	Developing countries	World
Rice									
2000 (mmt)	119.8	221.7	1.1	14.8	5.5	7.4	20.4	370.3	390.7
2050 No CC (mmt)	168.9	217.0	2.6	17.8	10.3	18.3	20.3	434.9	455.2
2050 No CC (% change)	41.0	–2.1	144.4	19.8	87.4	146.0	–0.3	17.4	16.5
CSIRO (% change)	–14.3	–8.1	–0.2	–21.7	–32.9	–14.5	–11.8	–11.9	–11.9
NCAR (% change)	–14.5	–11.3	–0.8	–19.2	–39.7	–15.2	–10.6	–13.6	–13.5
Wheat									
2000 (mmt)	96.7	102.1	127.5	23.5	23.6	4.5	205.2	377.9	583.1
2050 No CC (mmt)	191.3	104.3	252.6	42.1	62.0	11.4	253.7	663.6	917.4
2050 No CC (% change)	97.9	2.1	98.1	78.7	162.3	154.4	23.6	75.6	57.3
CSIRO (% change)	–43.7	1.8	–43.4	11.4	–5.1	–33.5	–7.6	–29.2	–23.2
NCAR (% change)	–48.8	1.8	–51.0	17.4	–8.7	–35.8	–11.2	–33.5	–27.4
Maize									
2000 (mmt)	16.2	141.8	38.0	80.1	8.2	37.1	297.9	321.3	619.2
2050 No CC (mmt)	18.7	264.7	62.7	143.1	13.1	53.9	505.1	556.2	1.061.3
2050 No CC (% change)	15.7	86.6	65.1	78.8	59.4	45.3	69.6	73.1	71.4
CSIRO (% change)	–18.5	–12.7	–19.0	–0.3	–6.8	–9.6	11.5	–10.0	0.2
NCAR (% change)	–8.9	8.9	–38.3	–4.0	–9.8	–7.1	1.8	–2.3	–0.4
Millet									
2000 (mmt)	10.5	2.3	1.2	0.0	0.0	13.1	0.5	27.3	27.8
2050 No CC (mmt)	12.3	3.5	2.1	0.1	0.1	48.1	0.8	66.2	67.0
2050 No CC (% change)	16.5	50.1	77.2	113.0	128.0	267.2	60.5	142.5	141.0
CSIRO (% change)	–19.0	4.2	–4.3	8.8	–5.5	–6.9	–3.0	–8.5	–8.4
NCAR (% change)	–9.5	8.3	–5.2	7.2	–2.7	–7.6	–5.6	–7.0	–7.0
Sorghum									
2000 (mmt)	8.4	3.1	0.1	11.4	1.0	19.0	16.9	43.0	59.9
2050 No CC (mmt)	9.6	3.4	0.4	28.0	1.1	60.1	20.9	102.6	123.5
2050 No CC (% change)	13.9	11.6	180.9	145.3	12.2	216.9	23.6	138.7	106.2
CSIRO (% change)	–19.6	1.4	–2.7	2.3	0.3	–2.3	–3.1	–2.5	–2.6
NCAR (% change)	–12.2	6.7	–10.4	4.3	0.7	–3.0	–7.3	–1.5	–2.5

In summary, and assuming little further capacity to expand agricultural land area, grain production per unit land area will need to more than double over this century to deal with rising population and dietary change under the climate change scenario (Long & Ort 2010).

Increase in average temperature could result in longer potential growing seasons at high latitudes, and often shorter seasons at low latitudes because of interactions with rainfall, evapotranspiration and soil moisture. The optimum climates for our major grains will move Pole–ward, such that the North American wheat and corn belts will move northwards into Canada, with parallel changes on the Eurasian steppe (Easterling *et al.* 2007). However, this does not mean that yields can be maintained simply by moving the production areas Pole-ward. These areas lack the high quality soils of the

prairie and steppe. At other locations, for example the wheat belt of Western Australia, Pole-ward movement is not possible since the ocean lies to the south.

The supply of freshwater is an absolute essential for all forms of agriculture, although the amount of water required varies greatly between different agricultural types and climatic regions. Given the scale of agricultural activity (1.54 billion ha of arable and permanent crops alone) (FAO 2009), this means that agricultural activity dominates the use of freshwater and accounts for some 70% of withdrawals from water resources globally (Fischer *et al.* 2007; Boutraa 2010). Climate change will have a direct impact on water availability for irrigated crops. During this century, climate change may further reduce water availability for global food production, as a result of projected mean changes in temperature and precipitation regimes (Rosenzweig *et al.* 2002). Clearly, making agriculture sustainable requires a major reduction in water use in many regions. The IPCC projected that the land area affected by drought will increase and water resources in affected areas could decline as much as 30% by mid-century (Christensen *et al.* 2007). Furthermore, socio–economic pressures over the next several decades will lead to increased competition between irrigation needs and demand from non-agricultural sectors, potentially reducing the availability and quality of water resources for food.

1.3. High temperature and water deficit effects on photosynthesis

Reduced precipitation and increased temperature will likely affect plant growth and development, primarily due to changes in the photosynthetic carbon assimilation (Reddy, Rasineni & Raghavendra 2010), which is the primary factor determining crop productivity and yield (Long & Ort 2010).

By disentangling down-regulation of photosynthesis under water and temperature stresses, the leaf capacity to fix CO_2 may become limited by diffusive and/or biochemical processes (Galmés, Medrano & Flexas 2007b). Deciphering which of these limitations are predominant would provide valuable information to pre-adapt crops and breed for drought- and heat-tolerant genotypes. Diffusional limitations result from the resistance to CO_2 diffusion from the atmosphere to intercellular leaf spaces (Flexas *et al.* 2008). Diffusion limitations are principally observed when plants close stomata, in a conservative response to decrease water losses under conditions of water scarcity (Flexas & Medrano 2002a). However, by closing stomata, the pathway through which atmospheric CO_2 enters the leaf is also restricted (Galmés *et al.* 2011a). Thus,

beyond stomata, a considerable body of evidence has been accumulated that leaf mesophyll conductance to CO_2 (g_m) also responds to changes in environmental conditions such as drought and temperature (Flexas *et al.* 2008; Warren 2008). In consequence, stomatal and mesophyll elements characterize the diffusion component of photosynthesis, and therefore are the determinants of the availability of CO_2 for the carboxylation reaction. The enzyme responsible to fix CO_2 in the photosynthetic organisms is Rubisco (ribulose–1,5–bisphosphate carboxylase/oxygenase), and constitutes the main biochemical or metabolic component limiting photosynthesis under most physiological conditions, particularly under non-saturating CO_2 concentrations and saturating irradiances (von Caemmerer 2000).

1.3.1. Effects of high temperature on photosynthesis

With the predicted increase in global air temperature, plant responses to increasing temperature have become a major area of concern (Gunderson, Norby & Wullschleger 2000; Rustad *et al.* 2001). Higher temperatures cause heat stress in plants, and under these conditions plants grow and produce less (Porter & Semenov 2005). In some cases, the decrease in crop productivity under warmer conditions is related to effects on the phenology and reproductive stages. Higher temperatures can drive shorter life cycles, resulting in less seasonal photosynthesis, shorter reproductive stage, and thus lower yield (Ainsworth & Ort 2010). Vegetative development is accelerated in cereals with increasing temperature, but it is the dramatically shorter grain-filling period with raising temperature that portends major consequences for yield (Lawlor & Mitchell 2000). Also, high temperature stress has been described to affect any of the reproductive processes, including pollen viability, female gametogenesis, pollen-pistil interaction, fertilization, and grain formation (Ainsworth & Ort 2010). However, under agriculture conditions, crop yield is frequently limited by carbon gain, and therefore, the response of photosynthesis to higher temperature will be a major driver of temperature effects on crops yield.

Photosynthesis is considered among the plant functions most sensitive to high temperature (Berry & Björkman 1980; Quinn & Williams 1985). The typical response of net CO_2 assimilation rate (A_N) to temperature (A_N–T curve) can be described by a peaked surface (Fitter & Hay 2002), with low A_N at cool temperatures, increasing to maximum rate at optimal temperatures and then decreasing again at very high temperatures (Berry & Björkman 1980). For example, Law & Crafts-Brandner (1999)

found that gradually increasing the leaf temperature from 35 to 42.4°C for cotton and from 30 to 39.2°C for wheat caused a 50% reduction in the net CO_2 assimilation. In consequence, temperatures out of the optimum results in a considerable loss of potential productivity (Lobell & Asner 2003). In principle, the shape of the A_N–T curve is determined by three primary sets of processes, namely biochemical, diffusive and respiratory processes. The response of respiration to changes in temperature will be treated in detail in section 1.4. Therefore, in the following, a short review of the effects of temperature on the biochemical and diffusive components of photosynthesis is described.

Regarding biochemistry, an increase in temperature reduces the photosynthetic efficiency by stimulating photorespiration (Ogren 1984; Brooks & Farquhar 1985) as well as by damaging the photosynthetic apparatus. The temperature induced decreases in the $[CO_2]/[O_2]$ ratio and in the specificity of Rubisco for CO_2 relative to O_2 ($S_{c/o}$), favour oxygenation of RuBP over carboxylation, increasing flux through photorespiration, which, together with increased rates of mitochondrial respiration, reduces the potential increase in CO_2 fixation at high temperature (Berry & Björkman 1980; Jordan & Ogren 1984; Kobza & Edwards 1987). However, the photosynthesis inhibition by high temperature occurs under both photorespiratory and non-photorespiratory conditions (Kobza & Edwards 1987; Crafts-Brandner & Salvucci 2000). Therefore, the reduction in photosynthesis cannot be simply explained by the greater rate of photorespiration at high temperatures, and other direct inhibitory effects on photosynthesis have been described (Kobza & Edwards 1987). Formerly, photosystem II (PSII) had often been regarded as the most affected by heat stress, but the evidence shows that Rubisco is even more sensitive to inactivation by moderate heat stress (Weis 1981; Kobza & Edwards 1987). The Rubisco limitation at high temperatures is due principally by decrease in activity. The activation state of Rubisco is regulated by Rubisco activase (Portis 2003), which may fail to maintain a high Rubisco activation state at high temperature because of its thermolability (Crafts-Brandner & Salvucci 2000; Salvucci & Crafts-brandner 2004). Rubisco activase is an AAA^+ protein that converts Rubisco sites from a closed, inactive conformation to an open and potentially active conformation (Andrews *et al.* 1995; Spreitzer & Salvucci 2002; Portis 2003). The closed conformation is stabilized and continually formed by the binding of certain sugar-phosphates that act as inhibitors of the enzyme (Andrews *et al.* 1995). Conversion from the closed to the open conformation not only frees Rubisco sites of

bound inhibitors, but is also required for spontaneous 'activation' by carbamylation and Mg^{+2} binding, prerequisites for productive RuBP binding and subsequent catalysis (Spreitzer & Salvucci 2002). In addition, there are others process than indirectly could restrict the Rubisco activity, for example, triose phosphate utilization limitation at moderately high temperature may result in lowered ATP/ADP ratios (Sharkey 1989). Moreover, moderately high temperature reduces the pH component of the trans-thylakoid proton motive force (Zhang *et al.* 2009) and lowered electron flow (Schrader *et al.* 2004). Such reductions in electron transport could reduce the stromal redox state (Ruuska *et al.* 2000; Yamori *et al.* 2011). A lower ATP/ADP ratio or stromal redox state can result in a decreased Rubisco activase activity. It is still under debate whether the reduced activity of activase is directly due to its intrinsic thermolability (van de Loo & Salvucci 1996; Salvucci & Crafts-brandner 2004; Hozain *et al.* 2010; Yamori *et al.* 2012) or to indirect effects on the electron transport rate (Campbell & Ogren 1990; Sharkey 2005; Sage, Way & Kubien 2008).

During photosynthesis, CO_2 has to move from the atmosphere surrounding the leaf to the sub-stomatal internal cavities through the stomata, and from there to the site of carboxylation inside the chloroplastic stroma through the leaf mesophyll (Flexas *et al.* 2008). The diffusion of CO_2 into the leaf and chloroplast is directly dependent on temperature via diffusivity effects, stomatal control, solubilization and membrane permeability (Sage & Kubien 2007). Depending upon species and growth conditions, stomata can open with rising temperature (a common response when vapor pressure deficit is low), close (often in response to increasing vapor pressure deficit with rising temperature) or remain unaffected (Kemp & Williams 1980; Monson *et al.* 1982; Tenhunen *et al.* 1984; Sage & Sharkey 1987; Santrucek & Sage 1996; Cowling & Sage 1998; Yamori *et al.* 2006). It is worth noting that the sensitivity of photosynthesis to variation in stomatal conductance generally increases at warmer temperatures, because the biochemical controls over photosynthesis at high temperature are more sensitive to changes in C_i (Sage & Kubien 2007). Therefore, stomatal limitations are generally greater at elevated temperature, regardless of the stomatal response (Sage & Sharkey 1987; Hendrickson *et al.* 2004). Mesophyll conductance (the conductance of CO_2 from the stomata to the chloroplast stroma) is also sensitive to many environmental factors, including temperature (Flexas *et al.* 2008) that can affect g_m within minutes to hours (Bernacchi *et al.* 2002; Warren & Dreyer 2006; Yamori *et al.* 2006; Warren 2008). For instance, in rice, plants had a threefold higher mesophyll conductance when are grown

and measured at 32°C relative to 25°C (Makino, Nakano & Mae 1994). Likewise, Scafaro *et al.* (2011) observed increases on g_m in three species of rice with the rise in the measuring temperature.

1.3.2. Effects of water deficit on photosynthesis

The rate of photosynthesis declines as plant water stress increases (Flexas & Medrano 2002b), and as occurred with high temperature, this decline may be a consequence of limitations in CO_2 diffusion and/or photosynthetic metabolism. While it is fairly well-established that under mild to moderate stress, drought induced reductions in photosynthesis are mainly due to diffusive limitations — i.e. decreased stomatal and mesophyll conductance — (Flexas & Medrano 2002a; Chaves *et al.* 2002; Galmés *et al.* 2007b; Flexas *et al.* 2012), there is less of a consensus about the limiting process when drought intensity is increased above mild stress, with some studies reporting metabolic impairment (Parry *et al.* 2002; Zhou, Lam & Zhang 2007), while others not (Sharkey & Seemann 1989; Tezara *et al.* 1999). Such apparent discrepancy may be explained by the severity and/or duration of the stress imposed (Flexas *et al.* 2004, 2006; Galmés *et al.* 2011a b). In addition, when the activity of Rubisco is impaired, the precise underlying mechanisms (i.e., decreased Rubisco content, activation state or presence of inhibitors) seem to depend on the species analyzed (Bota, Medrano & Flexas 2004; Galmés *et al.* 2013) and the quickness of water stress imposition (Flexas *et al.* 2006). Other authors have suggested that decreased Rubisco activity under water stress is a direct consequence of secondary oxidative stress, which in turn depends on the prevailing levels of irradiance (Zhou *et al.* 2007).

Biochemically, the metabolic limitations are suggested by the impaired ATP synthesis (Tezara *et al.* 1999; Flexas *et al.* 2004). It is because the decrease in ATP synthesis indirectly decreases the regeneration of ribulose–1,5–bisphosphate (RuBP), therefore limiting the rate of CO_2 fixation. However, particularly under drought, the CO_2 availability in leaves is strongly reduced because of decreases in g_s and g_m, and hence plants operate in a CO_2 range where Rubisco carboxylation is the primary metabolic rate limiting photosynthesis (von Caemmerer 2000). Therefore, despite claims that drought impairs chloroplast ATP synthesis and RuBP regeneration (Tezara *et al.* 1999; Lawlor & Tezara 2009), if any metabolic limitation occurs under drought, this is very likely to be mediated by Rubisco (Flexas *et al.* 2004; Grassi & Magnani 2005; Galmés *et al.* 2013).

1.3.3. Effects of varying temperature on Rubisco kinetics

Rubisco is the major leaf protein in plants (Sage & Pearcy, 1987), and it catalyzes the carboxylation (CO_2 addition) or oxygenation (O_2 addition) of ribulose-1,5-bisphosphate (RuBP) leading to the subsequent formation of two molecules of 3-phospho-d-glycerate (PGA; via the carboxylation reaction), or one molecule of PGA and one molecule of 2-phospho-glycolate (PG; via the oxygenation reaction O_2) (Gutteridge & Keys 1985). This bi-functionality, coupled with slow catalysis, leads to Rubisco frequently being the principal determinant of the efficiency with which autotrophs use CO_2, light, water, and mineral resources (Tcherkez, Farquhar & Andrews 2006).

The efficiency of photosynthesis is strongly dependent on the relative specificity of Rubisco for CO_2 versus O_2 ($S_{c/o}$), the enzyme's maximum carboxylase turnover rate (k_{cat}^c) and Michaelis-Menten constant for CO_2 (K_c), O_2 (K_o) and RuBP (K_{RuBP}), this last little studied regarding its temperature dependence (Laing, Ogren & Hageman 1974; Whitney, Houtz & Alonso 2011; Galmés *et al.* 2014). These Rubisco kinetic parameters vary with temperature, and ultimately, these responses may play an important role in mitigate the effects of climate change. The maximum carboxylase turnover rate (k_{cat}^c) and the Michaelis-Menten constant for CO_2 (K_c) increase with rising temperatures (Sage 2002). The CO_2/O_2 specificity factor ($S_{c/o}$) shows the opposite behavior, with a decrease at higher temperatures. Therefore, the increase in the carboxylase turnover rate at high temperatures does translate in an improvement in the photosynthetic capacity due to the decrease in Rubisco affinity for CO_2, this latter given by the increase in K_c and the decrease in $S_{c/o}$, and also the decreased in the CO_2/O_2 concentration ratio in solution that contributes to the Rubisco photorespiration in the C_3 plants (Hall & Keys 1983; Jordan & Ogren 1984).

Understanding the dependence of the Rubisco kinetic parameters to the different environmental conditions is critical, since these parameters and the maximum Rubisco activity are important determinants of leaf CO_2 exchange, and are fundamental components of leaf models of photosynthesis (Farquhar, von Caemmerer & Berry 1980; von Caemmerer & Farquhar 1981). However, in most studies using the photosynthetic models, the Rubisco kinetics parameters measured in the transgenic *Nicotiana tabacum*, L. cv W38 has been popularized as standard values used for the rest of species (von Caemmerer 2000; Bernacchi *et al.* 2001, 2002), which assumed that the species-specific

differences are to be negligible (Yamori & von Caemmerer 2009; Galmés *et al.* 2011a; Greer & Weedon 2012; Scafaro *et al.* 2012). However, recent studies point to the importance of considering the species-specific response to temperature of Rubisco kinetics to make more accurate the photosynthetic models (Diaz-Espejo 2013; Walker *et al.* 2013). It is therefore imperative to clarify if differences in the temperature dependencies of the Rubisco kinetics parameters are related to the thermal environment to which the species is adapted, and also if they depend on the species photosynthetic mechanism. For the latter, it is necessary to examine the functional differences in Rubisco from C_3 and C_4 plants between plants that are closely related phylogenetically, in order to unravel key residues potentially governing the thermal responsiveness of Rubisco kinetics (Kubien *et al.* 2008). Understanding such puzzles is necessary to identifying solutions for improving Rubisco catalysis that, in coordination with other photosynthesis-enhancing approaches, are aimed at delivering a second "green revolution" to increase productivity in globally important C_3–grain crops such as wheat and rice (Evans & von Caemmerer 2011).

1.4. Effect of water deficit and high temperature on respiration, carbon balance and biomass allocation

Plant productivity depends on the balance between photosynthesis and respiration (Lambers, Chapin & Pons 1998). While photosynthesis occurs only in the green tissues during the light period, mitochondrial respiration (R) occurs continuously in every cell of the organism. Therefore, it is not surprising that 30-80% of daily photosynthetic carbon gain is released into the atmosphere by plant R. In other words, from the 100 Gt of atmospheric CO_2 being annually fixed by Rubisco (Field *et al.* 1998), it is estimated that plant R releases 60 Gt C $year^{-1}$ (Field 2001).

By providing the driving force for biosynthesis, cellular maintenance and active transport in plants, R is an essential process, which can be down-regulated, but not completely suppressed, even under the most intense and detrimental stress. Zero R simply means 'no life'. In consequence, under certain circumstances, particularly when photosynthesis is largely suppressed (such as water and temperature stresses), R becomes the most important factor controlling productivity (Flexas *et al.* 2005; Lambers & Ribas-Carbó 2005).

There is little information about the effects of water stress on plant R. Flexas *et al.* (2005) suggested a biphasic response on the relationship between R and the leaf relative water content, with an initial phase of decline in R at mild intensities of stress – associated to decrease in the growth component of respiration –, followed by an increase in R at more severe intensities – associated with the maintenance component of respiration. However, data from Galmés *et al.* (2007c; 2011a) did not support the existence of such biphasic response of respiration to drought imposition. In turn, they showed that increase in R occurs under conditions where drought severely restricts CO_2 availability inside the leaves, after a g_s threshold coincident with that observed to induce down-regulation of the activity of many other metabolic components. Regardless of this discrepancy, it is clear that A_N/R drops as water stress progresses (Galmés *et al.* 2007c).

There is more information and consensus about the absolute effects of temperature on R; respiration increases exponentially with temperature (e.g. see Atkin *et al.* 2005). Typically, the response of R to temperature has been described by changes in the parameter Q^{10}, which is defined as the proportional change in R per 10ºC increase in temperature (Atkin & Tjoelker 2003). Variation in Q^{10} has been observed among species from different thermal origins, but also within a single species depending on the tissue, and the growth and measurement temperatures (e.g. Atkin *et al.* 2005; Wright *et al.* 2006). Overall, because of the different temperature dependencies of A_N and R, the A_N/R may change with switches in temperature (Atkin, Scheurwater & Pons 2006; Campbell *et al.* 2007).

In summary, quantitatively, both water and heat stress can induce changes in A_N/R, and therefore in the leaf – and indirectly plant – carbon balance. In addition, qualitatively alterations, in the sense of changes in the carbon allocation patterns, have been also described under water and temperature stresses (Poorter & Nagel 2000; Galmés *et al.* 2005; Atkin *et al.* 2006). For example, water deficit reduces the number of leaves per plant and individual leaf size (Hussain *et al.* 2008). Drought-induced reduction in leaf area is ascribed to suppression of leaf expansion through reduction in photosynthesis (Rucker *et al.* 1995), and results in a lower evaporative surface (Ludlow 1989). Hence, under water stress, plants usually allocate a higher proportion of biomass to the underground tissues, i.e., increased root/shoot ratio (Boyer 1982; Galmés *et al.* 2005; Vile *et al.* 2012). Still associated with water stress, plants usually present a higher leaf mass per unit leaf area (LMA), due to increased leaf density and/or thickness (Galmés, Medrano & Flexas 2007a; Poorter *et al.* 2009; Vile *et al.* 2012). LMA is an

important component of plant *strategy* and reflects changes in key photosynthetic traits such as g_m (Flexas *et al.* 2008). Alterations in the biomass allocation have been also observed in response to changes in temperature (Boese & Huner 1990; Xu & Huang 2001; Atkin *et al.* 2006). Typically, long-term exposure to moderately high temperatures result in plants exhibiting an increased investment in the shoot (i.e., decreased root/shoot ratio) and leaves with lower LMA (Potter & Jones 1977; Woodward 1979; Friend & Woodward 1990; Loveys *et al.* 2002; Luomala *et al.* 2005; Poorter *et al.* 2009), in contrast to the drought-induced changes (see above). However, according to literature data, the effects of temperature on the biomass accumulation and allocation greatly differ amongst species and temperature regimes at which plants are grown (Atkin *et al.* 2006; Nagai & Makino 2009).

1.5. Plant acclimation or adaptation?

Plant ecophysiology and crop physiology provide multiple examples of the detrimental effects of environmental stresses – among them drought and high temperature – on plant performance (Schulze, Beck & Müller-Hohenstein 2002). To re-adjust their performance, plants respond to such changes in their growth conditions, in different ways. Responses over time scales, covering multiple generations of a population, and therefore involving evolutionary changes adapting a population to a modified environment, are named adaptation. Precisely, the term adaptation defines processes conferred by genetic attributes that serve to fit the plant to ambient conditions, in this case, ambient temperature and/or water availability (Alscher & Cumming 1990). By contrast, short-term adjustments in response to transient changes in the environment conditions are named acclimation.

For comparison, acclimation is important for the survival of individual plants in rapidly changing environments, while adaptation is important for species, or at the least populations (or genotypes) survival in the long–term. Acclimation is to meteorology what adaptation is to climate. Within minutes, acclimation relies on changes in preexisting components within a biochemical pathway (e.g. increase in the activity of Rubisco in response to increase in temperature). This short–term acclimation changes are easily reversible. Acclimation in a longer term (typically within days or weeks following an environmental change) involves altered patterns of gene expression, reallocation of resources and morphological changes. These responses are not

immediately reversible and often lead to the development of a different phenotype. Other authors preferred to name static or developmental acclimation to those changes not easily reversible, and dynamic acclimation to changes that can be reserved in the short–term (Walters 2005; Athanasiou *et al.* 2010). Finally, adaptive responses are consequence of changes in the gene sequences. Adaptive and acclimation changes may involve different scale processes, from subcellular level to adjustments in whole plant architecture (Athanasiou *et al.* 2010).

An important appreciation of data from literature is that the ability to adapt and acclimate to a given stress varies between species and even between genotypes of the same species, which suggests that these processes must carry advantages with some cost (Athanasiou *et al.* 2010). For instance, developmental acclimation consisting in higher LMA after decreased water availability may have the pay-off of lower photosynthetic capacity due to decreased g_s and g_m (Medrano, Flexas & Galmés 2009; Galmés *et al.* 2012). Related to this, some authors have raised the idea that the ultimate objective of acclimation is homeostasis, i.e. to fully reserve the detrimental consequences of the environmental change and thereby maintaining optimal performance (Walters 2005; Atkin *et al.* 2006). Hence, homeostasis forms the basis of methods used to assess the degree of acclimation (for a review, see Atkin *et al.* 2005).

The term plasticity is inherently integrated in the acclimation response (Valladares, Gianoli & Gómez 2007). By definition, phenotypic plasticity is the environmentally sensitive production of alternative phenotypes by given genotypes (DeWitt & Scheiner 2004). The perfect plasticity would therefore explain that the theoretical maximum fitness is achieved by expressing the best phenotype in each environment. The perfect plasticity is an insuperable strategy.

The present thesis will focus in explore the long-term acclimation, by growing plants under different conditions of temperature and water availability; and short-term acclimation, by measuring plants under different conditions of temperature. Adaptation and acclimation potential and selection for adaptive and acclimation traits will be important in developing future crops with superior performance under environmental stress.

1.6. Drought and heat stress combination

In the field, crops are routinely subjected to a combination of different abiotic stresses (Heyne & Brunson 1940; Moffat 2002). Drought and heat stress indeed represent an excellent example of two abiotic stresses occurring in the field simultaneously, particularly under Mediterranean conditions (Craufurd & Peacock 1993; Jiang & Huang 2001). Data from researchers, farmers and public institutions demonstrate that the combination of drought and heat stress had significantly greater detrimental effect on the growth and productivity of some crops – like corn, barley and sorghum – compared with each of the two stresses applied individually (Savage & Jacobson 1935; Heyne & Brunson 1940; Craufurd & Peacock 1993; Perdomo, Murphy & Berkowitz 1996; Jiang & Huang 2001). The extent of the economic damage caused by a combination of drought and heat stress represents can be dramatic. For example, in USA, co-occurrence of drought and heat stress caused damage costing more than US$4.2 billion in August 2000 (National Climatic Data center).

However, because the majority of abiotic stress studies performed under controlled conditions are focused to a single stress (Machado & Paulsen 2001; Zhang *et al.* 2007; Vile *et al.* 2012), a considerable gap exists between the knowledge gained by these studies and that required to develop plants with enhanced tolerance to field conditions where co-occurrence predominate. This is not surprising that transgenic lines developed with enhanced tolerance to a particular stress failed to show enhanced tolerance when tested in the field (e.g. McKersie, Bowley & Jones 1999; Gao, Qian & Miao 2000). Therefore, it is essential to focus on the morphological, physiological and biochemical aspects of heat and drought stress combination to bridge this gap and to facilitate the development of crops with enhanced tolerance to field conditions.

The morphological, physiological and biochemical processes set in motion by a specific stress, either drought or heat, likely differ from those activated by a combination of these two stresses, which might require an unique acclimation response customized to each stress (Mittler 2006). Combined drought and heat stress might require conflicting or antagonistic responses, for example, during heat stress plants open stomata to cool their leaves by transpiration. However, if heat stress occurs at time with water shortage, then plants will not be able to open stomata and the leaf temperature will inevitably increase (Rizhsky, Liang & Mittler 2002). In consequence, an appropriate acclimation response to co-occurring drought and heat stress needs to compensate for the existing antagonistic aspects of the stress combination.

At the molecular level, it is logical to assume that the simultaneous exposure to different abiotic stress conditions will result in the co-activation of different stress-response pathways, in addition to those pathways specific to each particular stress (Rizhsky *et al.* 2004). Cross-talk between co-activated pathways is likely to be mediated at different levels (Chaves, Flexas & Pinheiro 2009; Pinheiro & Chaves 2011), including transcription factors (Cardinale, Casini & Arrhenius 2002; Xiong & Yang 2003), stress hormones (Anderson *et al.* 2004), calcium (Bowler & Fluhr 2000), reactive oxygen species (ROS) signaling (Mittler *et al.* 2004), and receptors or signaling complexes (Casal 2002).

In summary, the extent of damage caused to agriculture by the combination of drought and heat stresses underscores the need to develop crops with enhanced tolerance to the combination of these two stresses. This enhanced tolerance is not the simply the sum the acclimation responses set individually to drought and heat stress. Thus, to develop transgenic crops with enhanced tolerance to field conditions, we need to invest more efforts to study the combinatory effects of drought and heat stress.

Chapter 2

OBJECTIVES AND OUTLINE

2.1. GENERAL OBJETIVES

As stated in the Introduction, high temperature (HT) and water deficit (WD) are two of the environmental factors that most limit photosynthesis and plant growth. Besides, climate change is predicted to increase global temperatures altering also the precipitation patterns, which leads to long drought periods in some world regions. Under these detrimental prognostics, it is predicted a highest limitation in plant growth that finally trigger in an impaired to agricultural productivity. Therefore, it is imperative to increase our knowledge on the processes governing the photosynthetic responses to heat and drought stresses.

The general objectives of the present work were: (1) to identify effect of high temperature and water deficit on the main physiological processes related to plant carbon balance in three of the world's most important crops, (2) to study the role of Rubisco on the plant response processes to high temperature and water deficit.

2.2. SPECIFIC OBJECTIVES

1. To determine the effects of long-term growth under HT and WD on the leaf carbon balance and water use efficiency and their limitations to the total biomass production.
2. To compare the single and combined effects of HT and WD on the growth, physiological and biochemical responses in plants of rice, wheat and maize.
3. To unravel whether the acclimation capacity of the physiological processes in rice, wheat and maize to HT and WD is related to their original climate and photosynthetic mechanism.
4. To analyze the responses of leaf carbon assimilation, leaf respiration and the leaf CO_2 diffusive capacity under long–term HT and WD.
5. To determine the improvement in the accuracy of the C_3 and C_4 photosynthetic models after incorporation of species–specific kinetics of Rubisco and PEPC.
6. To analyze the performance of Rubisco and Rubisco activase under long–term growth HT, WD and their combination HT–WD and to relate them with their relative contributions to biochemical and diffusive limitations to photosynthesis in rice, wheat and maize.
7. To determine whether Rubiscos from closely related species with different photosynthetic mechanism have different temperature dependence.

2.3. OUTLINE OF THE THESIS

The contents of this thesis are organized in 8 Chapters that cover the study of responses and acclimation of physiological and biochemical processes in plants of rice, wheat and corn, as well as the study of the thermal responses of the Rubisco kinetic parameters in related plants of *Flaveria* with different photosynthetic mechanism.

Chapter 1: INTRODUCTION

This Chapter introduces the background and sets the contexts for this Thesis. It includes a general overview of the current and future impacts of the climate change on agriculture. Also, this describe the limitations exerted by high temperature and water deficit on plant growth and physiological and biochemical processes.

Chapter 2: OBJECTIVES AND OUTLINE

In this Chapter the general and specific objectives are presented, as well as a brief outline of the Thesis. Finally, the publications derived from this Thesis are listed.

Chapter 3: PLANT GROWTH AND PHYSIOLOGICAL ACCLIMATION UNDER HIGH TEMPERATURE AND WATER DEFICIT

This chapter presents the effects of long-term high temperature and water deficit on plant growth and biomass allocation of rice, wheat and maize, with a special emphasis in the response of leaf carbon balance and the intrinsic water use efficiency.

Chapter 4: PHOTOSYNTHETIC LIMITATIONS UNDER THE INDIVIDUAL AND COMBINED EFFECTS OF HIGH TEMPERATURE AND WATER DEFICIT

This chapter focuses on analyze the physiological responses of photosynthesis under long-term high temperature and water deficit stresses and its acclimation under these detrimental conditions. Likewise, this chapter determines the improvement in the accuracy of the C_3 and C_4 photosynthetic models after incorporation of species-specific kinetics of Rubisco and PEPC.

Chapter 5: RUBISCO AND RUBISCO ACTIVASE PLAY AN IMPORTANT ROLE IN THE BIOCHEMICAL LIMITATIONS OF PHOTOSYNTHESIS IN RICE, WHEAT AND MAIZE UNDER HIGH TEMPERATURE AND WATER DEFICIT

This chapter covers the effects of high temperature and water deficit on Rubisco and Rubisco activase and their inference in the biochemical limitations to photosynthesis.

Chapter 6: TEMPERATURE DEPENDENCE OF RUBISCO KINETICS IN RELATED SPECIES OF *FLAVERIA*

This chapter describes the thermal responses of the Rubisco kinetics parameters, and its activation energies, from related species of *Flaveria* with different photosynthetic mechanisms C_3, C_3/C_4 and C_4.

Chapter 7: GENERAL DISCUSSION

This chapter contains a general discussion and overview of all the results presented in Chapters 3, 4, 5 and 6.

Chapter 8: CONCLUSIONS

The last Chapter presents a list of the main conclusions derived from the present Thesis in relation to the general objectives described in Chapter 2.

Chapter 3

PLANT GROWTH AND PHYSIOLOGICAL ACCLIMATION UNDER HIGH TEMPERATURE AND WATER DEFICIT

3.1. EFFECTS OF LONG-TERM INDIVIDUAL AND COMBINED WATER AND TEMPERATURE STRESS ON THE GROWTH OF RICE, WHEAT AND MAIZE: RELATIONSHIP WITH MORPHOLOGICAL AND PHYSIOLOGICAL ACCLIMATION

Juan Alejandro Perdomo, Miquel À. Conesa, Hipólito Medrano, Miquel Ribas-Carbó, Jeroni Galmés[†]

Research Group on Plant Biology under Mediterranean Conditions. Universitat de les Illes Balears, Ctra. de Valldemossa Km.7.5, Palma 07122, Spain.

[†]**Corresponding author:** Jeroni Galmés

e-mail: jeroni.galmes@uib.cat

fax: +34 / 971 / 173184

tel: +34 / 971 / 252710

Abstract

The present study evaluates the long-term individual and combined effects of high temperature (HT) and water deficit (WD) stress on plant growth, leaf gas-exchange and water use efficiency in cultivars of the three most important crops worldwide, rice, wheat and maize. Plant biomass accumulation (B_t) decreased under all treatments, being the combined HT-WD treatment the most detrimental in all three species. Although decreases in B_t correlated with adjustments in biomass allocation patterns (i.e., the leaf area ratio), most of the variation observed in B_t was explained by changes in leaf gas exchange parameters. Thus, integrated values of leaf carbon balance obtained from daily course measurements of photosynthesis and respiration were better predictors of plant growth than the instantaneous measurements of leaf gas exchange. Leaf water use efficiency, assessed both by gas exchange and carbon isotope measurements, was negatively correlated with B_t under WD, but not under the combined WD and HT treatment. A comparative analysis of the negative effects of single and combined stresses on the main parameters showed an additive component for WD and HT in rice and maize, in contrast to wheat. Overall, the results of the specific cultivars included in the study suggest that the species native climate plays a role shaping the species acclimation potential to the applied stresses. In this regard, wheat, originated in a cold climate, was the most affected species, which foretells a higher affectation of this crop due to climate change.

Abbreviations

A_N, net CO_2 assimilation rate; B_t, total plant biomass; C_a, atmospheric CO_2 concentration; CT, control temperature; $\delta^{13}C$, carbon isotopic composition; dA_N, daily net CO_2 assimilation rate; dA_N/dg_s, daily average intrinsic water use efficiency; dg_s, daily stomatal conductance; dR, daily leaf dark respiration; F_m, maximum fluorescence; F_o, background fluorescence; F_v/F_m, maximum quantum efficiency of PSII; GRC, growth response coefficient; GRC_{LAR}, growth response coefficient for the leaf area ratio; GRC_{LCB}, growth response coefficient for the leaf carbon balance; HT, high temperature; iA_N, instantaneous net CO_2 assimilation rate; iA_N/ig_s, mid-morning instantaneous water use efficiency; ig_s, instantaneous stomatal conductance; iR, instantaneous leaf dark respiration; LA, leaf area; LAR, leaf area ratio; LCB, leaf carbon balance; LMA, leaf mass area; LMR, leaf mass ratio; PPFD, photosynthetic

photon flux density; RAI, relative affectation index; RWC, relative water content; WD, water deficit; WW, well watered.

Introduction

The increasing global demand for agricultural products causes the need to almost double crop yield in the next 50 years (Foresight 2011). This enhancement of production needs to be particularly notorious for those crops with the highest contribution to human food: rice, wheat and maize (FAO 2010). Compounding the problem, continued emissions of greenhouse gases are causing important climatic changes in the main agricultural areas worldwide (Rowlands et al. 2012, Wheeler and von Braun 2013). Although certain consequences of climate change could promote specific crop gains in particular regions, the overall impacts on agriculture are expected to be negative, chiefly as a consequence of higher growing season temperatures and increased frequency and severity of drought periods (Lobell et al. 2008, Nelson et al. 2009, Ainsworth and Ort 2010, Long and Ort 2010). Given these negative predictions, there is an urgent need to determine, for the main crops worldwide, growth patterns and physiological performance under detrimental environmental conditions.

Under most stressful conditions, including water deficit and high temperatures, crop production is frequently limited by the net carbon gain (McCree 1986). This is because photosynthesis and respiration are extremely sensitive to both water scarcity and high temperatures (Flexas et al. 2005, Gratani et al. 2008, Stitt 2013). Furthermore, since carbon intake is unavoidably linked to water loses through stomata, it implicitly determines water use efficiency, a parameter of paramount importance under water and temperature stress conditions (Flexas et al. 2013, Zhao et al. 2013). Under these stressful conditions, nitrogen metabolism is also affected (Yousfi et al. 2012, Li et al. 2013), compromising nitrogen use efficiency and, ultimately, plant growth.

There is abundant information on the effects of water and temperature stresses on key processes governing photosynthesis and respiration (Mittler 2006, Prasad et al. 2011). Water deficit causes a decrease in net CO_2 assimilation rate (A_N), mainly as a consequence of restricted access of CO_2 to the Rubisco catalytic sites in the chloroplast (Flexas et al. 2012). This limited CO_2 availability for carboxylation results in diminished photosynthetic efficiency due to higher photorespiration rates (Voss et al. 2013). Decreased Rubisco concentration observed in some species under water deficit further compromises a positive leaf carbon balance (Parry et al. 2002, Galmés et al.

2013). In turn, despite contradictory results (for a review see Atkin and Macherel 2009), literature data agree in that the inhibitory effect of water deficit on the mitochondrial respiration rate is quantitatively lower than the effects caused by similar intensities of stress on the photosynthetic CO_2 assimilation rate (Flexas et al. 2005, Galmés et al. 2007a, Atkin and Macherel 2009). Thus, enhancing the relative importance of respiration in governing plant productivity under drought (Atkin and Macherel 2009, Escalona et al. 2012). Since leaf carbon balance dictates the amount of photoassimilates available for other plant tissues, and overall crop production, knowledge of the effects of water deficit on respiration will help to understand the effect of such stress on the whole plant growth. Hence, alteration of the leaf carbon balance due to stressful conditions may be linked to decreases in growth at the whole plant level and thus, knowledge on the effect of drought on such balance is pivotal (Flexas et al. 2006, Escalona et al. 2012, Zhao et al. 2013).

Regarding to temperature, heat stress has global effects on plant metabolism, reducing plant growth. There are alterations of mitochondrial functions such as production of reactive oxygen species and secondary metabolite accumulation, with direct impact on water relations, transpiration and CO_2 solubility, and on the photosynthetic machinery and enzyme activity (reviewed in Wahid et al. 2007). In the chloroplast, changes in nitrogen content and/or partitioning into photosynthetic enzymes have been related to thermal ambient (Yamori et al. 2009), and decreased Rubisco activity has been reported under elevated temperatures (Carmo-Silva et al. 2012). In mitochondria, an increase in temperature results in an immediate increment of the respiration rate (Atkin and Tjoelker 2003). Such enhancement in the rate of respiratory CO_2 release, coupled with inhibited CO_2 assimilation above the thermal optimum, decreases the leaf and plant carbon balance and severely impacts plant growth at high temperatures (Atkin et al. 2006).

Most well-documented responses of photosynthesis and respiration to water and temperature stresses correspond to short-term experiments, where environmental conditions are rapidly modified and the physiological response of plants is typically monitored within hours to days. However, field stress normally develops much more gradually, allowing plants to develop acclimation responses through changes in gene expression patterns (Galmés et al. 2007b, Shinozaki and Yamaguchi-Shinozaki 2007, Pinheiro and Chaves 2011). Indeed, plant responses measured in short-term experiments may respond mainly to rapid physiological alterations rather than physiological

acclimation, i.e., changes leading to similar or enhanced plant physiological performance under the new conditions (reviewed in Way and Yamori 2014). There is abundant data demonstrating that acclimation is generally more complete when plants develop tissues under the new environmental conditions (Loveys et al. 2003, Atkin et al. 2006, Campbell et al. 2007, Way and Yamori 2014). The acclimation potential, however, may be influenced by the genetic background of each species and thus, derived from its native climate conditions (Hikosaka et al. 2006, Nagai and Makino 2009, Yamori et al. 2010). Thus, the response of photosynthesis and respiration to high temperature may differ between plants from cool and warm habitats (Berry and Björkman 1980, Yamori et al. 2010), as it does the response of these two physiological processes to drought between plants from humid and dry habitats (Galmés et al. 2012, Smith and Dukes 2013). Similarly, the performance of photosynthesis and respiration under water and heat stress depends on the species specific photosynthetic mechanism (Flexas et al. 2006, Smith and Dukes 2013, Yamori et al. 2014). According to these evidences, it should be expected that species with contrasting native climatic conditions and photosynthetic mechanisms present different sensitivity to drought and heat stresses and, also, different acclimation potential. Ignoring the acclimation potential can result in an over-estimation of the effects of drought and temperature stresses on photosynthesis and respiration, especially over long-term periods (Flexas et al. 2009).

Notoriously, most studies on the effect of high temperature and drought on plant performance consider only one of the stresses. However, under natural conditions both stresses are frequently linked, which is the prevalent scenario in arid and semi-arid regions worldwide, i.e., higher temperature leads to higher water scarcity, and also higher plant water demand to maintain growth rates (Long and Ort 2010). Therefore, it is important to consider the additive or interactive effects of combined water and high temperature stresses, as they may significantly differ from those caused by the single stress (Rizhsky et al. 2004, Aranjuelo et al. 2009, Pérez et al. 2011, Vile et al. 2012).

In the present study, we assessed the individual and combined effects of high temperature (HT) and water deficit (WD) on leaf physiology and plant growth in fully acclimated plants of rice, wheat and maize. These three species were selected as being the three most important crops in terms of worldwide production (FAO 2010) and because of their different climatic origin (temperate for wheat, and warm for rice and maize) and photosynthetic mechanism (C_3 for rice and wheat, and C_4 for maize). In particular, the objectives of the present study were: i) to quantify the effects of long-

term growth under HT and WD on plant biomass production, ii) to factorize the stress-driven decreases in biomass with changes in plant morphological traits, iii) to relate limited growth capacity with unbalances in the leaf carbon balance and the intrinsic water use efficiency, iv) to compare the additive effects of the combined WD × HT stress with those of the individual stresses, and v) to investigate whether the acclimation capacity of these crop species is related to their original climate and photosynthetic mechanism.

Materials and methods

Plant material, growth conditions and treatments

Rice (*Oryza sativa* L. cv. Bomba), spring wheat (*Triticum aestivum* L. cv. Cajeme) and maize (*Zea mays* L. cv. Carella) were grown from seeds in a greenhouse in 3.5 L pots containing a 70:30 mixture (by vol.) of horticultural substrate (Projar S.A, Spain) and perlite (granulometry A13, Projar S.A, Spain). After 2 weeks, seedlings were selected to uniform size with one plant per pot in maize, and ten plants per pot in wheat and rice. Thereafter, plants were moved to a walk-in-growth chamber (phytotron) under controlled conditions of light intensity, photoperiod, relative humidity and temperature. Light was provided by metal halide lamps (OSRAM, Germany) placed at specific distances from the plants to obtain a photosynthetically active photon flux density (PPFD) of 500 µmol m^{-2} s^{-1}, with a photoperiod of 12 h day/12 h night. Ambient temperature and relative humidity were monitored with portable sensors Testo 175-H1 data logger (Gerilab, Spain). Relative humidity was maintained between 40 - 60% using humidifiers. Assays were performed in two separate rounds of experiments with two plants batches of similar age. The first batch was performed at control temperature (CT, 25/20°C; VPD, 1.8/1.0 kPa day/night), and the second batch at high temperature (HT, 38/33ºC; VPD, 3.5/2.3 kPa day/night). Only temperature and VPD differed between batches while all other environmental conditions (e.g. light intensity and quality, air removal, photoperiod duration) were constant and computer controlled.

Ten pots per species were grown at field capacity until they presented fully expanded leaves (typically two weeks) per each temperature regime. Thereafter, twenty days after germination, pots of all species were randomly assigned to two different irrigation treatments: five pots per species were maintained at field capacity throughout the experiment (well watered – WW – control plants) and five were maintained at 45%

field capacity (moderate water deficit treatment, WD), as determined by daily pot weight compensating the daily water losses with 50% Hoagland's solution. All measurements and samples were taken at least forty days after the water treatment was initiated (i.e., 60 days after germination) on new leaves developed completely under the temperature and/or water treatments.

Leaf relative water content, leaf area and leaf mass area

Leaf relative water content (RWC, calculated as [Fresh weight – Dry weight]/[Turgid weight – Dry weight]) was determined at mid-morning, following the procedures described in Galmés et al. (2007c). Whole plant leaf area (LA) was determined in fresh leaves using an AM-300 Area Meter (Analytical Development Co., UK). Leaf mass area (LMA) was calculated as the ratio of dry mass to leaf area. A total of five replicates were obtained per species × treatment combination.

Growth parameters

All plants were harvested 70 days after germination (i.e., 50 days after water treatment was applied). Plant fractions – leaves, stems and roots – were separated to measure dry weight by drying in a ventilated oven at 60ºC until constant weight. Based on these data, total plant dry biomass (B_t) and leaf mass ratio (LMR) were calculated. Leaf area ratio (LAR) was calculated as the ratio of plant leaf area to B_t. Five replicates were measured for each species × treatment combination.

Instantaneous and daily course measurements for gas exchange and chlorophyll a fluorescence

Leaf gas exchange and chlorophyll *a* fluorescence measurements were performed with a Li-6400 (Li-Cor Inc., USA) on the youngest fully expanded leaf at leaf temperature similar to growth temperature (25ºC or 38ºC).

Instantaneous net CO_2 assimilation (iA_N) and stomatal conductance (ig_s) were measured at mid-morning at a PPFD of 1,500 µmol m^{-2} s^{-1} (provided by the light source of the Li-6400 with 10% blue light), a CO_2 concentration in the leaf chamber (C_a) of 400 µmol CO_2 mol^{-1} air and a relative humidity between 40 and 60%. The instantaneous leaf dark respiration rate (iR) was obtained at predawn at a C_a of 400 µmol CO_2 mol^{-1} air.

Background (F_o) and maximum (F_m) fluorescence signal were also determined at predawn with a leaf chamber fluorometer (Li-6400-40, Li-Cor Inc., USA). F_o was measured at light intensity of ca. 0.5 µmol photon m^{-2} s^{-1} set at a frequency of 600 Hz. F_m was measured with a saturating PPFD pulse of ca. 8,500 µmol photon m^{-2} s^{-1} for 0.8 s. The maximum quantum yield of PSII (F_v/F_m) at predawn was calculated as, $F_v/F_m = (F_m-F_o)/F_m$.

For the daily course measurements, leaf gas exchange parameters were monitored during an entire day (24 h) through automated discrete measurements undertaken every 10 min with a Li-6400 equipped with a Sun+Sky leaf cuvette, i.e., at ambient PPFD of about 500 µmol m^{-2} s^{-1} and C_a of 400 µmol CO_2 mol^{-1} air. Daily average net CO_2 photosynthetic rate (dA_N), stomatal conductance (dg_s) and respiration rate (dR) were calculated from the integration of discrete measurements taken during the light (for dA_N and dg_s) or dark periods (for dR). Leaf carbon balance (LCB; mol CO_2 m^{-2} day^{-1}) was calculated as the integrated value of leaf net CO_2 assimilation rate over 24 h period.

Carbon isotope composition in leaf dry matter

Young leaves of rice, wheat and maize developed under all treatments, were taken at same moment of the harvest. Leaf samples were dried in an air-forced oven for 48 h at 60ºC, and ground to powder using a ball-mill (F-Kurt Retsch MM 200, Germany). Subsamples of 2 mg were analysed for carbon isotope ratio ($\delta^{13}C$) as long-term indicator of water use efficiency. Subsamples were combusted in an elemental analyser (Thermo Flash EA 1112 Series, Italy), and CO_2 was separated by chromatography and directly injected into a continuous-flow isotope ratio mass spectrometer (Thermo Delta Plus XP, Germany). Peach leaf standards (NIST 1547) were run every eight samples. $\delta^{13}C$ was calculated as: $\delta^{13}C$ sample (‰) = [(R sample/R standard) – 1] x 1000 (Farquhar and Richards 1984). $\delta^{13}C$ values are referred to a Pee Dee Belemnite standard.

Statistical analysis

Statistical significance of trait variation was tested by factorial ANOVA, with species, irrigation treatments and growth temperatures as fixed factors, and the interaction between treatments. Post hoc comparison between treatments was performed

with Duncan test ($P < 0.05$) using Statistica 6.0 software package (StatStof Inc., USA). Regressions coefficients were calculated with the 11.0 Sigma Plot software package.

To determine the change in LAR and LCB scaled with respect to the relative change in B_t, the growth response coefficient (GRC) of these two parameters were calculated according to Poorter and Nagel (2000):

$$GRC_{LAR} = \frac{d\ LAR}{LAR} \Big/ \frac{d\ B_t}{B_t}$$

$$GRC_{LCB} = \frac{d\ LCB}{LCB} \Big/ \frac{d\ B_t}{B_t}$$

A relative affectation index (RAI) was calculated to analyze the effects of temperature and water availability on each species. This index was calculated dividing the sum of the significance level for the ten parameters analyzed (P_i) by the potential maximum significance under each stress (P_{max}). The significance level was considered 1 when $P < 0.05$, 2 $P < 0.01$ and 3 $P < 0.001$, being $P_{max} = 30$ per species on each stress. The larger RAI, the larger the effect of the stress on the corresponding species.

$$\text{RAI} = \frac{\Sigma\ P_i}{\Sigma\ P_{max}}$$

Results

Stress indicator parameters

Maximum quantum yield of PSII at predawn (F_v/F_m) and leaf relative water content (RWC) are characteristic parameters to evaluate the intensity of stress on plant physiological status (Flexas and Medrano 2002). According to F_v/F_m, wheat was the most affected species, with decreases under the individual (CT-WD and HT-WW) and combined stress (HT-WD) as compared to control treatment (CT-WW) (Table 1). In maize, F_v/F_m only decreased under CT-WD, while in rice the maximum efficiency of PSII did not decrease under any treatment. The response of leaf RWC to the applied treatments was similar to F_v/F_m, with decreases in wheat and maize under CT-WD and HT-WD, and no decreases in rice with respect to CT-WW (Table 1).

Table 1. Maximum quantum yield of PSII at pre-dawn (F_v/F_m) and mid-morning leaf relative water content (RWC), for plants grown at control (CT) and high temperature (HT) under well-watered (WW) and water deficit (WD) conditions. Values are means ± S.E. (n=5). Different letters denote statistically significant differences by Duncan analysis ($P < 0.05$) among treatments within each species.

Parameter	Rice				Wheat				Maize			
	CT-WW	CT-WD	HT-WW	HT-WD	CT-WW	CT-WD	HT-WW	HT-WD	CT-WW	CT-WD	HT-WW	HT-WD
F_v/F_m	0.76±0.03^{a}	0.76±0.1^{a}	0.76±0.02^{a}	0.78±0.02^{a}	0.82±0.1^{c}	0.79±0.01^{b}	0.77±0.01^{a}	0.78±0.01^{ab}	0.78±0.1^{b}	0.71±0.02^{a}	0.77±0.01^{b}	0.76±0.01^{b}
RWC (%)	90.0±0.9^{ab}	89.0±1.6^{a}	90.0±1.5^{ab}	93.0±0.9^{b}	94.1±0.2^{c}	86.5±2.4^{a}	93.0±0.8^{bc}	90.0±1.4^{ab}	95.0±1.4^{c}	88.0±1.9^{b}	92.0±1.0^{bc}	81.0±1.9^{a}

Total plant biomass and morphological parameters

In all three species, total plant biomass (B_t) was reduced under both CT-WD and HT-WW treatments, with rice being most affected under HT-WW, maize under CT-WD and wheat equally under the two single stresses (Fig. 1A). However, in all three species, the combined stress HT-WD was the most detrimental treatment in terms of growth inhibition, with a decrease of ca. 60% in maize and rice and ca. 90% in wheat.

Compared to control, there was a significant reduction of total plant leaf area (LA) under all stress treatments in wheat and maize, but only under the combined stress in rice (Fig. S1A). Among the individual treatments, LA was more sensitive to CT-WD than to HT-WW in wheat, while the contrary was observed in maize. In spite of the decrease in LA, leaf mass ratio (LMR) increased under all treatments in wheat, under HT-WW and HT-WD in rice and under CT-WD in maize (Fig. 1B).

The response of the leaf mass area ratio (LMA) to the different stresses was species-dependent (Fig. 1C). In rice, LMA decreased under HT-WW and HT-WD, but not under CT-WD. In wheat, LMA was also reduced under HT-WW, but increased under CT-WD and was not affected under the combined stress. Finally, in maize LMA did not change under any of the treatments.

The described changes in LMR and LMA induced important alterations in the leaf area ratio (LAR) in rice and wheat, particularly under HT-WW and HT-WD treatments (Fig. 1D). In maize, LAR was significantly altered only under CT-WD, as compared to CT-WW.

Among the plant morphological parameters, decreases in B_t induced by the treatments were mostly related to decreased LA and increased LAR in the three crops when data of all treatments within each species was compiled ($P < 0.05$, Fig. S1). B_t also correlated negatively with LMR in rice and wheat, and positively with LMA in rice. These trends between B_t and the morphological parameters were maintained, with few exceptions, when comparing CT-WW individually with CT-WD, HT-WW and HT-WD within each species (data not shown).

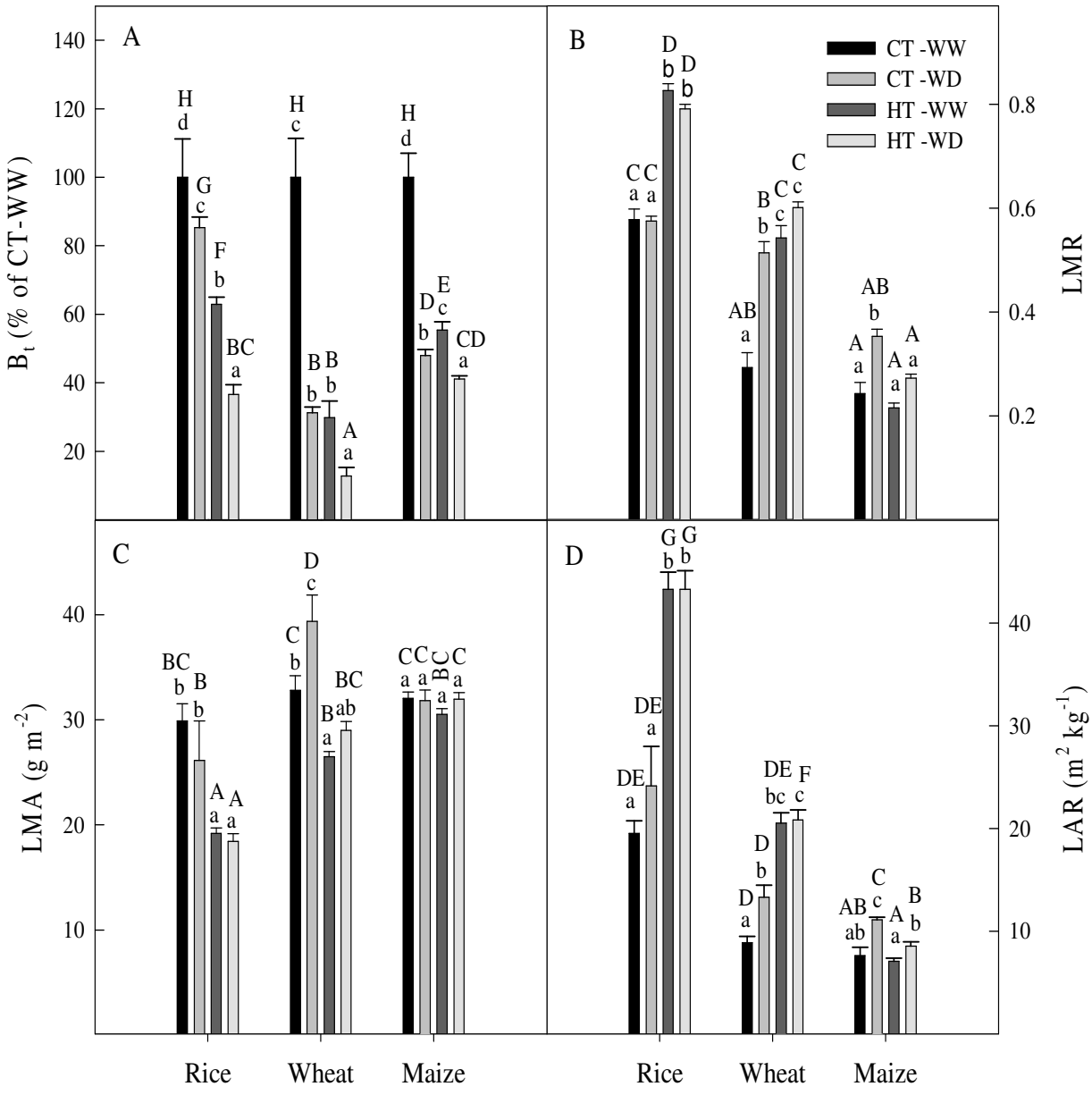

Figure 1. (A) Total plant biomass (B_t), (B) leaf mass ratio (LMR), (C) leaf mass area (LMA) and (D) leaf area ratio (LAR), for each species grown at control (CT) or high temperature (HT), under well-watered (WW) and water deficit (WD) conditions. Values are means ± S.E. (n=5). Different uppercase and lowercase letters denote statistically significant differences by Duncan analysis ($P < 0.05$) among species × treatment interactions and among treatments within each species, respectively.

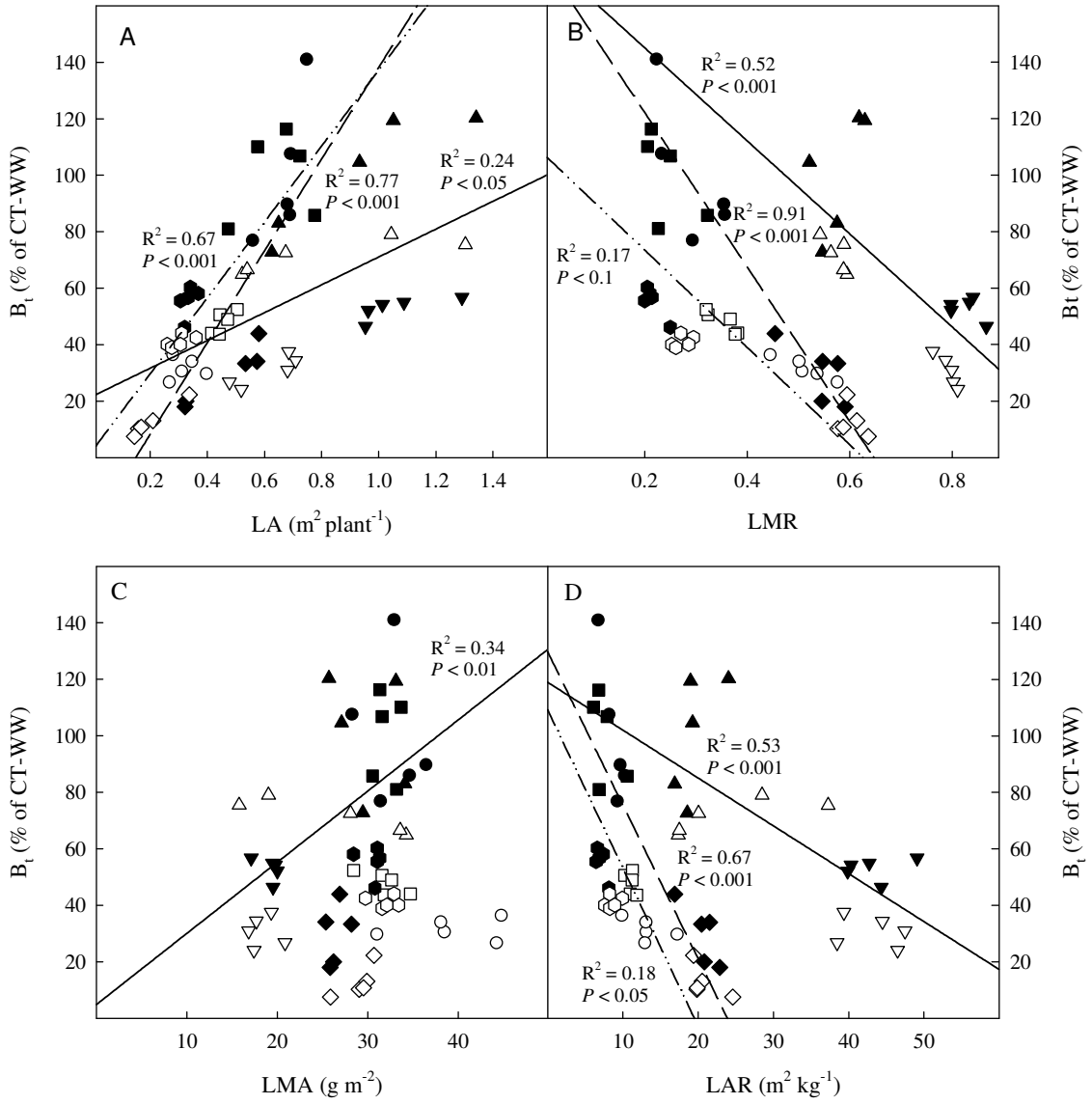

Figure S1. The relationship between the total plant dry biomass (B_t) and (A) the leaf area (LA), (B) the leaf mass ratio (LMR), (C) the leaf mass area (LMA) and (D) the leaf area ratio (LAR), for the three species. Symbols as follows: ▲ CT-WW rice, △ CT-WD rice, ▼ HT-WW rice, ▽ HT-WD rice, ● CT-WW wheat, ○ CT-WD wheat, ◆ HT-WW wheat, ◆ HT-WD wheat, ■ CT-WW maize, ■ CT-WD maize, ⬢ HT-WW maize, ⬢ HT-WD maize. The solid regression line is for rice, the dashed regression line is for wheat and the dashed-dotted regression line is for maize.

Instantaneous rates of leaf photosynthesis and mitochondrial respiration

Under CT-WW, the three crops presented similar values, around 25 μmol m^{-2} s^{-1}, for the instantaneous rates of photosynthetic CO_2 assimilation (iA_N), measured at mid-morning under light-saturating conditions (Fig. 2A). As expected, under CT-WW the instantaneous stomatal conductance (ig_s) was lower in the C_4 maize than in C_3 rice and

wheat (Fig. 2C). Growing under CT-WD provoked a decrease of iA_N in all three species. By contrast, compared to CT-WW, HT-WW produced a significant decrease of iA_N only in wheat (Fig. 2A). The combined stress resulted in iA_N reductions similar to CT-WD in rice and wheat, thus without additive effects; whereas no significant differences were observed between CT-WW and HT-WD in maize, suggesting a palliative effect of HT on the negative impact by WD in this species. In general, treatment-induced changes in iA_N followed those changes observed in ig_s, indicative of the predominant role of stomatal limitations on CO_2 assimilation (Fig. 2A, C).

Stress-induced changes of the instantaneous rates of leaf mitochondrial respiration (iR) were smaller than those observed for iA_N, with the exception of wheat under HT-WW (Fig. 2E). Within each species, iR increased under CT-WD, compared to CT-WW, only in wheat; while all species increased iR when grown under HT-WW. The combined stress treatment ameliorated the HT-driven increase in iR, and iR under HT-WD was higher than the rates measured under control treatment only in maize (Fig. 2E).

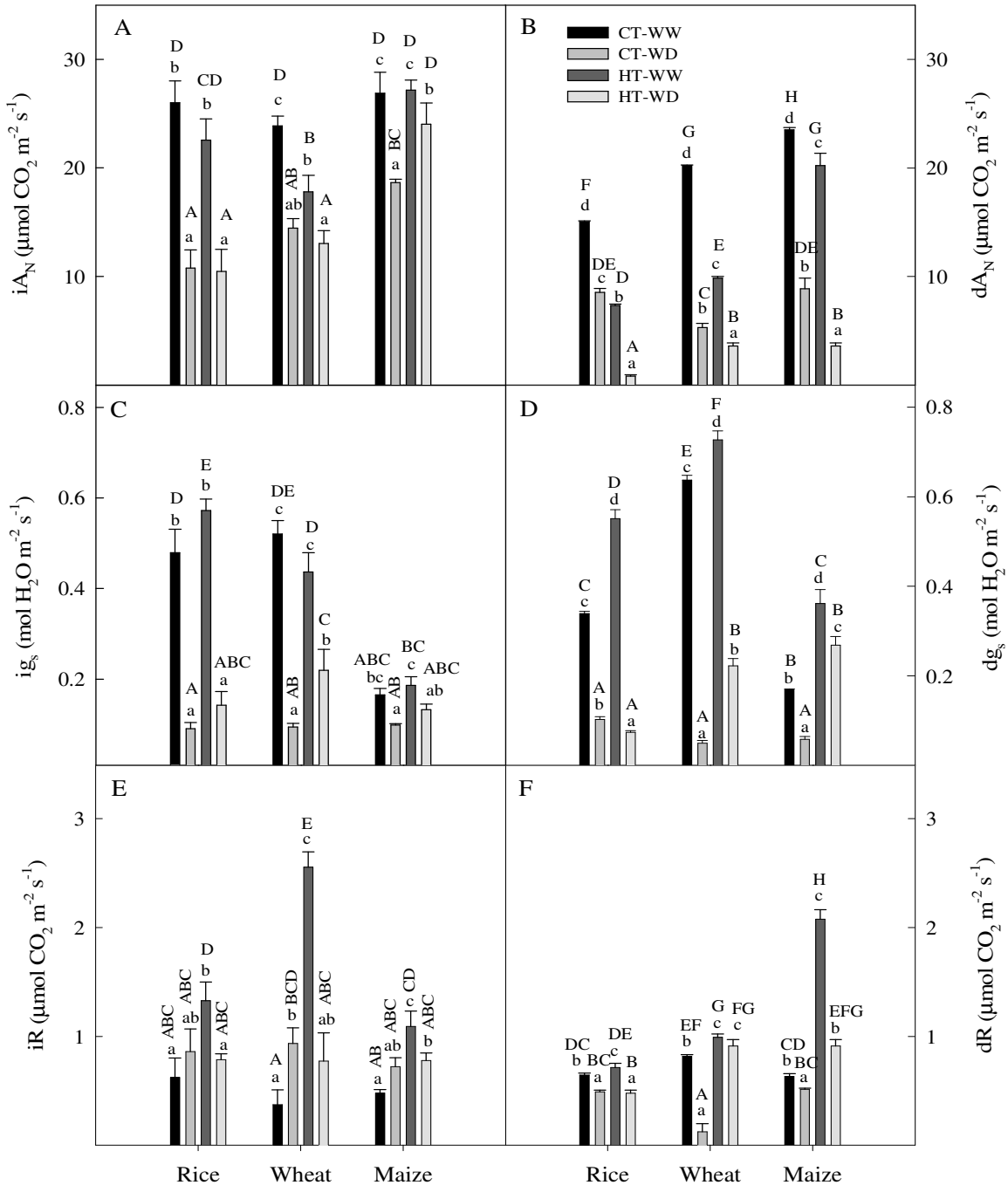

Figure 2. (A, B) The mid-morning instantaneous (iA_N) and daily average net CO_2 assimilation rate (dA_N), (C, D) the mid-morning instantaneous (ig_s) and daily average stomatal conductance (dg_s), (E, F) the pre-dawn instantaneous (iR) and night average mitochondrial respiration (dR), for each species grown at control (CT) or high temperature (HT), under well-watered (WW) and water deficit (WD) conditions. Values are means ± S.E. (n=5). Different uppercase and lowercase letters denote statistically significant differences by Duncan analysis ($P < 0.05$) among species × treatment interactions and among treatments within each species, respectively.

Daily course of leaf photosynthesis and mitochondrial respiration

The response of the daily average net CO_2 assimilation rate (dA_N) to the applied treatments was similar to that of iA_N in wheat, whereas in rice and maize the patterns displayed by iA_N and dA_N differed for some treatments. For instance, rice and maize decreased dA_N, but not iA_N, under HT-WW as compared to CT-WW (Fig. 2A, B). This discrepancy is explained by the constant decline in net CO_2 assimilation rate over the light-period in these two species when grown under HT-WW (i.e. negative slope A_N *vs*. time; $P < 0.001$). Alternatively, differences between dA_N and iA_N may be attributable to species-specific trends in the photosynthetic response to variable light intensities (500 and 1500 μmol photons m^{-2} s^{-1} for dA_N and iA_N, respectively). Overall, changes in dA_N paralleled those observed in B_t (compare Figs. 1A and 2B), and thus, dA_N was more explicative of the treatment-induced effects on biomass accumulation than iA_N, especially in rice and maize (Table S1). In turn, ig_s and dg_s showed similar patterns of response to the different stress treatments in the three species (compare Fig. 2C, D).

Table S1. Correlation coefficients between the total plant biomass (B_t) and the mid-morning instantaneous (iA_N) and the daily average net CO_2 assimilation rate (dA_N), the pre-dawn instantaneous (iR) and night average mitochondrial respiration rate (dR), and the leaf carbon balance (LCB). For each species, data of all treatments were considered together. The significance level of each correlation is indicated with * when $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

	Rice	Wheat	Maize
iA_N	0.46*	0.82***	0.46*
dA_N	0.88***	0.90***	0.66**
iR	-0.44	-0.40	-0.41
dR	0.33	0.00	-0.20
LCB	0.88***	0.91***	0.68***

Under CT-WW, the over-night average rates of mitochondrial respiration (dR) were higher than the predawn instantaneous rates of iR in wheat and maize, but not in rice (Fig. 2E, F). The effect of WD on dark respiration rates differed between iR and dR: while iR was unaffected or increased, dR decreased in plants grown under CT-WD compared to CT-WW in all three species. Similarly, the effect of HT-WW on dR was

different to that described for iR, while the trend for additive effects of the combined stresses on iR persisted in the three crops.

Plant biomass accumulation and leaf carbon balance

The leaf daily carbon balance (LCB) was calculated as the integrative value of the leaf CO_2 photosynthetic assimilation and respiratory CO_2 release over 24 h. Among the three species under CT-WW, rice and maize showed the lowest and the highest values for LCB, respectively (Fig. 3). When grown under HT-WW, LCB decreased in all three crops, with wheat presenting the highest reduction and maize the lowest. LCB also decreased in plants grown under CT-WD, with wheat and maize showing the highest decrements. The combined stress treatment was by far the most detrimental in terms of LCB decrease in the three crops. All three species displayed a positive relationship between LCB and B_t ($P < 0.05$; Fig. 3).

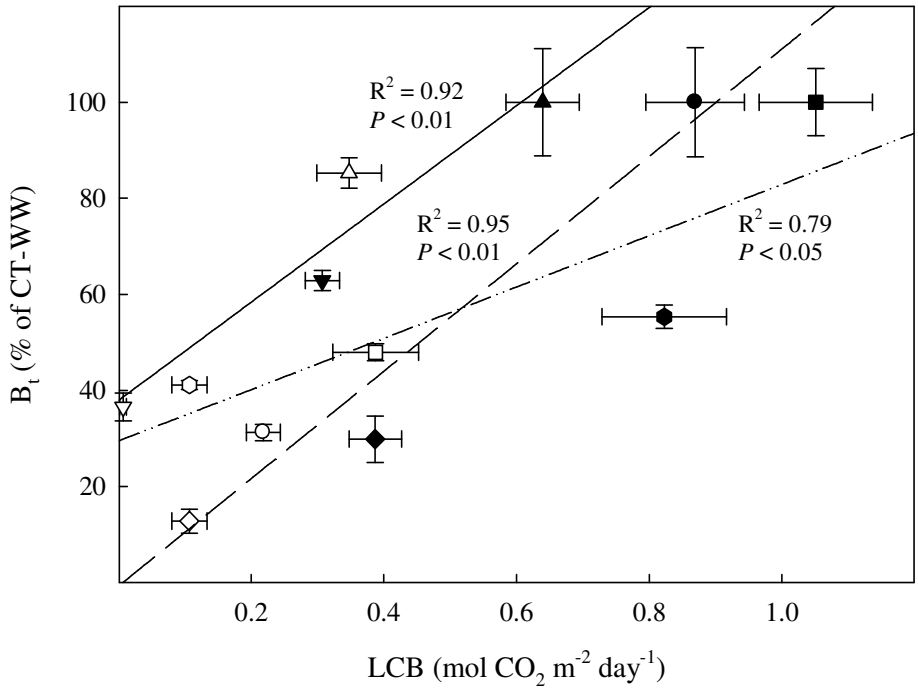

Figure 3. The relationship between the total plant dry biomass (B_t) and the leaf carbon balance (LCB), for the three species. Symbols for species and treatments as follows: ▲ CT-WW rice, △ CT-WD rice, ▼ HT-WW rice, ▽ HT-WD rice, ● CT-WW wheat, ○ CT-WD wheat, ◆ HT-WW wheat, ◆ HT-WD wheat, ■ CT-WW maize, ■ CT-WD maize, ⬢ HT-WW maize, ⬢ HT-WD maize. The solid regression line is for rice, the dashed regression line wheat and the dashed-dotted regression for maize.

Analysis of the growth relative components for leaf area ratio (GRC_{LAR}) and leaf carbon balance (GRC_{LCB}) confirmed that decreases in B_t were generally associated with

an increase in LAR and a decrease in LCB (Table 2). Wheat followed this general trend under all treatments, while rice and maize presented some exceptions. In particular, decreased B_t in CT-WD rice was only significantly related to decreased LCB, and decreased B_t in HT-WW maize could not be explained by changes in LAR or LCB (Table 2).

Table 2. Growth response coefficient of plant biomass for the leaf area ratio (GRC_{LAR}) and the leaf carbon balance (GRC_{LCB}) in each of the species studied under water deficit (CT-WD), high temperature (HT-WW) and combined stress (HT-WD) treatments, with respect to the control treatment (CT-WW). The significance level of the relationship between total plant biomass and LAR and LCB is also indicated as * when $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Species	Treatment	GRC_{LAR}	GRC_{LCB}
Rice	CT-WD	-0.4	1.9*
	HT-WW	-1.3**	1.2***
	HT-WD	-0.7***	3.3***
Wheat	CT-WD	-0.3**	0.8***
	HT-WW	-0.7***	0.6***
	HT-WD	-0.4***	0.9***
Maize	CT-WD	-0.5***	1.0***
	HT-WW	0.1	0.4
	HT-WD	-0.1	2.2***

Leaf intrinsic water use efficiency and carbon isotopic composition

The leaf intrinsic water use efficiency, as the ratio between net CO_2 assimilation rate (A_N) and stomatal conductance (g_s), was calculated from both light-saturated instantaneous measurements (iA_N/ig_s) and daily average values measured under light chamber conditions (dA_N/dg_s). As expected, C_4 maize presented higher values of iA_N/ig_s than C_3 species under all treatments (Fig. 4A). Under CT-WD, all three species increased iA_N/ig_s, being this increase higher in rice and, particularly, in wheat. HT-WW did not affect iA_N/ig_s in wheat and maize, but decreased iA_N/ig_s in rice. Compared to CT-WW, HT-WD caused iA_N/ig_s increments in rice and wheat, although they were significantly lower than those observed under the single water deficit treatment (CT-WD). When calculated on daily average values, dA_N/dg_s confirmed the highest intrinsic

water use efficiency of maize, as well as the increase in C_3 plants under CT-WD (Fig. 4B). Notorious differences were observed between the response of iA_N/ig_s and dA_N/dg_s to high temperature and combined stresses. In all three species, dA_N/dg_s severely decreased in plants grown under HT-WW and HT-WD due to increased dg_s and decreased dA_N, respectively (Fig. 2D, F).

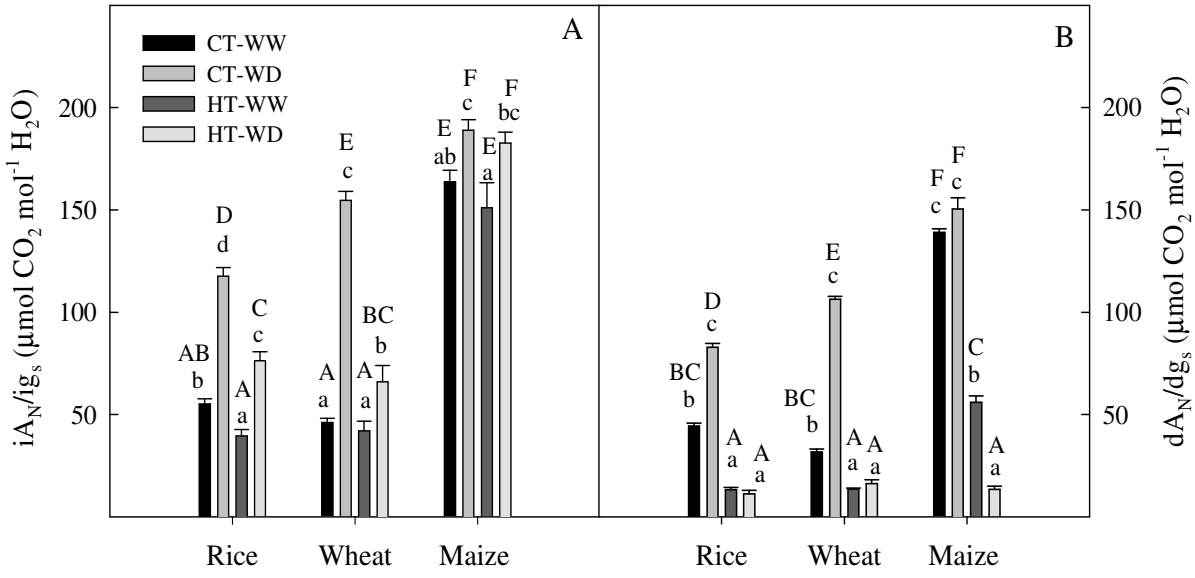

Figure 4. (A) The mid-morning instantaneous (iA_N/ig_s) and (B) daily average intrinsic water use efficiency (dA_N/dg_s), for each species grown at control (CT) or high temperature (HT), under well-watered (WW) and water deficit (WD) conditions. Values are means ± S.E. (n=5). Different uppercase and lowercase letters denote statistically significant differences by Duncan analysis ($P < 0.05$) among species × treatment interactions and among treatments within each species, respectively.

The observed changes in the intrinsic water use efficiency in rice and wheat were further corroborated by their carbon isotopic composition of leaves ($\delta^{13}C$), being the relationship between $\delta^{13}C$ and dA_N/dg_s significant in C_3 species ($P < 0.05$; Fig. 5A). As expected, $\delta^{13}C$ did not correlate with dA_N/dg_s for maize (Fig. 5B). In rice and wheat, increase in dA_N/dg_s under CT-WD was related to decreases in B_t (Fig. 6A), indicative of a trade-off between water use efficiency and growth. This trade-off was not observed when comparing CT-WD and HT-WD data. Instead, HT-WD-induced adjustments in B_t correlated positively with changes in dA_N/dg_s in both crops (Fig. 6B). In maize, no significant correlation was observed between dA_N/dg_s and B_t when comparing CT-WW and CT-WD plants, but a positive correlation between these parameters was also found when CT-WD and HT-WD plants were compared (Fig. 6).

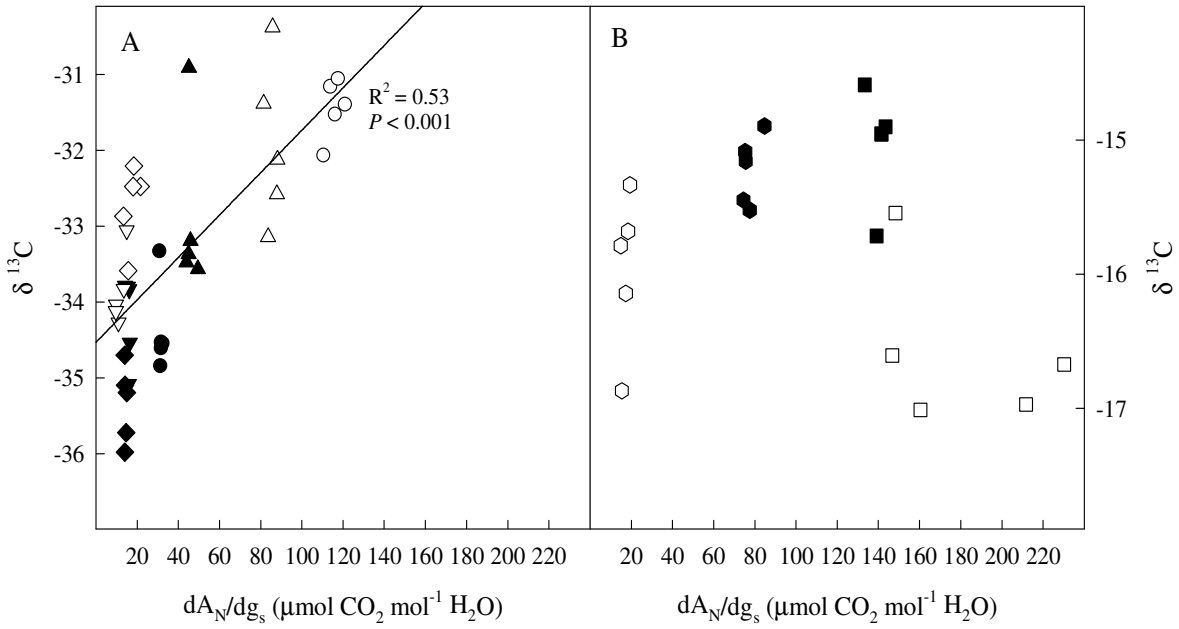

Figure 5. The relationship between the daily average intrinsic water use efficiency (dA_N/dg_s) and the leaf carbon isotopic discrimination ($\delta^{13}C$) in rice and wheat (A) and in maize (B). Symbols for species and treatments as follows: ▲ CT-WW rice, △ CT-WD rice, ▼ HT-WW rice, ▽ HT-WD rice, ● CT-WW wheat, ○ CT-WD wheat, ◆ HT-WW wheat, ◆ HT-WD wheat, ■ CT-WW maize, ■ CT-WD maize, ⬢ HT-WW maize, ⬢ HT-WD maize. The regression line in A is common for rice and wheat (y = 0.03x – 34.5).

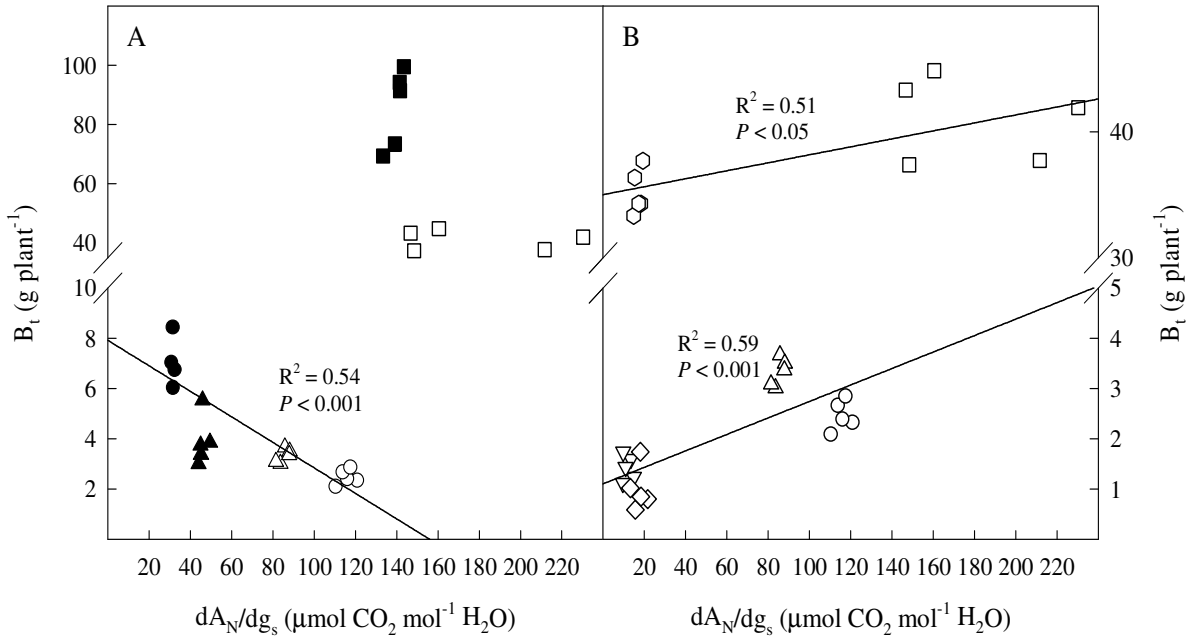

Figure 6. The relationship between the daily average intrinsic water use efficiency (dA_N/dg_s) and the total dry plant biomass (B_t) in (A) plants grown under control conditions (CT-WW) and water deficit (CT-WD), and (B) plants grown under water deficit (CT-WD) and combined stress treatment (HT-WD).

Symbols for species and treatments as follows: ▲ CT-WW rice, △ CT-WD rice, ▼ HT-WW rice, ▽ HT-WD rice, ● CT-WW wheat, ○ CT-WD wheat, ◆ HT-WW wheat, ◆ HT-WD wheat, ■ CT-WW maize, ■ CT-WD maize, ⬣ HT-WW maize, ⬣ HT-WD maize. In A, the regression line is common for rice and wheat ($y = –0.05x + 7.9$). In B, two regression lines are shown, one for C_4 maize ($y = 0.03x + 35.0$) and one for C_3 rice and wheat together ($y = 0.02x + 1.1$).

Discussion

In the present work, plants of rice, wheat and maize were grown under different combinations of long-term changes of irrigation (100% and 45% of field capacity) and temperature conditions (25/20°C and 38/33ºC) to assess their acclimation potential. Stress imposition started 20 days after germination and measurements were undertaken on fully acclimated leaves (i.e., those that emerged and developed after the application of the treatments). It is important to note that differences in growth temperature were not fully compensated by changes in atmospheric humidity. Therefore, significant differences in vapour pressure deficit were recorded between temperature treatments: 1.8/1.0 kPa day/night at control temperature (CT, 25/20°C) and 3.5/2.3 kPa at high temperature treatment (HT, 38/33ºC). Therefore, indirect effects of VPD on the photosynthetic performance cannot be ruled out on the thermal treatments.

Decreases in F_v/F_m and RWC caused by the drought and temperature changes were lower than those previously observed by Galmés et al. (2007c) and Hu et al. (2010) (Table 1). This apparent discrepancy may be attributed to acclimation mechanisms in leaves fully developed under stress, compared to short term responses (Campbell et al. 2007).

Decreases in biomass after long-term growth under WD and HT stress are partially explained by adjustments in plant morphological parameters

Compared to the control (CT-WW), all the stress treatments reduced total plant biomass (B_t) in all three crops (Fig. 1A), with higher impact of the combined stress. This result confirms recent findings (Prasad et al. 2011) and highlights the importance of considering the interactive effects of these two stresses to correctly assess biomass reductions in crops grown under stressful conditions. Although the extend of affectation by individual stresses on B_t was species-dependent, wheat presented the highest reduction in B_t under any of the treatments (Fig. 1A). The higher susceptibility of wheat to HT, as compared to rice, has already been documented (Nagai and Makino 2009), as

well as the higher acclimation potential to HT of C_4 species compared to C_3 species (e.g., reviewed in Yamori et al. 2014).

Changes in plant growth under stress have been factorized into morphological and/or physiological components that determine the plant's carbon economy (Lambers et al. 1989, Poorter and Nagel 2000, Galmés et al. 2005). The morphological component is related to the amount of leaf area per plant mass or the leaf area ratio (LAR), which in turn depends on two components: leaf mass per unit area (LMA) and a measure of biomass allocation (leaf mass ratio, LMR) (Konings 1989, Poorter and Van der Werf 1998). In species adapted to drought, LMA typically increases in plants subjected to water stress (Galmés et al. 2005, Poorter et al. 2009). In the present study, only wheat increased LMA in response to WD (Fig. 1C). In plants grown under HT, LMA decreased in rice and wheat (Fig. 1C), in contrast previously reported results (Nagai and Makino 2009). Differences between the present study and literature results may be due to cultivar specific responses or to the differences in light irradiance and temperature regime during plant growth (Atkin et al. 2006, Poorter et al. 2010). A positive correlation between B_t and LA was found in all species when data of all treatments was considered together (Fig. S1A). The decrease in the evaporative surface likely alleviated the intensity of stress, particularly under WD, as indicated by the minor changes observed in leaf RWC and F_v/F_m (Table 1). However, B_t correlated negatively with both LMR and LAR, indicating that adjustments in their relative allocation of biomass between different plant fractions influenced the growth capacity of plants under stress (Figs. 1 and S1). Kurimoto et al. (2004) and Nagai and Makino (2009) reported positive trends between relative growth rate (RGR) and LAR in wheat and rice grown at different temperatures. This apparent discrepancy could be attributed to the inclusion of low temperature stress in Kurimoto et al. (2004) and Nagai and Makino (2009), and to the combined effects of water deficit and heat stress in the present study, further emphasizing the importance of considering the interaction between different stresses occurring simultaneously.

Differences between the instantaneous and daily measurements of leaf gas-exchange parameters and their relation with biomass production

Important decreases in both instantaneous (ig_s) and daily average (dg_s) stomatal conductances were observed in plants grown under CT-WD and HT-WD (Fig. 2C, D), indicating that under drought stress plants decrease water losses via stomatal closure

(Flexas and Medrano 2002). By contrast, dg_s increased in plants grown under HT-WW, particularly in rice and maize (Fig. 2D). Increased dg_s may be advantageous to facilitate evaporative leaf cooling in well-watered plants developed at HT. Remarkably, wheat and maize plants grown under HT-WD also presented higher dg_s than CT-WD (Fig. 2D). A recent report has shown that *Arabidopsis* plants exposed to HT display increased transpiration and leaf cooling by temperature-mediated alterations in stomatal and plant architecture (Crawford et al. 2012).

Contrarily, for the daily average net CO_2 assimilation rate (dA_N) there was a notorious decrease in plants under stress compared to control in all species and treatments (Fig. 2B). Only maize under HT-WW approached homeostasis, in agreement with previous results in maize (e.g., Crafts-Brandner and Salvucci 2002) and as widely recognized for C_4 species (Berry and Björkman 1980). However, regarding the instantaneous net CO_2 assimilation rate (iA_N), full homeostasis was observed in rice and maize, but not in wheat, under HT-WW (Fig. 2A). This is in accordance with its higher sensitivity to high temperatures as compared to maize and rice (Wahid et al. 2007). Decreased light-saturated photosynthetic rates above the thermal optimum has been related to biochemical limitations, in particular, to increased photorespiration, lower concentration of Rubisco and decreased ability of Rubisco activase to maintain high Rubisco activation state (Keys 1999, Crafts-Brandner and Salvucci 2000, Kubien and Sage 2008). *In vitro* characterization of the temperature response of Rubisco and Rubisco activase would permit deciphering which of these processes is responsible for the improved photosynthetic performance in maize and rice, as compared to wheat. Under water deficit, the concomitant decreases in g_s and A_N observed in all species (Fig. 2) are indicative of predominant stomatal limitations (Flexas and Medrano 2002).

The above described changes in A_N and g_s lead to adjustments in their intrinsic water use efficiency (A_N/g_s) under different treatments (Fig. 4). In general, the observed response in the instantaneous measurements of the intrinsic water use efficiency (iA_N/ig_s, Fig. 4A) was in agreement with literature, with higher values in C_4 maize as compared to C_3 rice and wheat (Wang et al. 2012), a significant increase in rice and wheat plants grown under CT-WD (Ehdaie et al. 1991, Centritto et al. 2009), and non-significant changes in HT-WW-grown plants (Evans and von Caemmerer 2013). Remarkably, in rice and wheat iA_N/ig_s was higher for HT-WD than for HT-WW plants. Hence, WD could counteract the negative effects of HT on the intrinsic water use efficiency due to higher decreases in ig_s relative to iA_N (Fig. 2A, C).

The trends observed for the daily average measurements of intrinsic water use efficiency (dA_N/dg_s) resembled those described for iA_N/ig_s in response to water stress imposition (Fig. 4). However, plants of the three species grown under high temperature stress, either under well-watered (HT-WW) or water-deficit (HT-WD), presented important decreases in dA_N/dg_s, but not in iA_N/ig_s, compared to CT-WW. This discrepancy between dA_N/dg_s and iA_N/ig_s was particularly notorious in maize grown under HT-WW and HT-WD, and is related to the different irradiance levels during instantaneous and daily gas leaf exchange measurements and to intraday variations in A_N and g_s trends (data not shown).

The observed adjustments in the intrinsic water use efficiency were corroborated by the carbon isotope composition of leaves ($\delta^{13}C$), supporting the positive correlation between $\delta^{13}C$ and dA_N/dg_s in rice and wheat (Fig. 5A) (Farquhar et al. 1989, Wright et al. 1994). These correlations extrapolate conclusions based on daily gas-exchange measurements to long-term periods during the leaf life span of the C_3 crops (Seibt et al. 2008).

According to literature, increases in water use efficiency are usually achieved at the expense of some reductions in the photosynthetic CO_2 assimilation and, in consequence, their growth potential (Flexas et al. 2010, Galmés et al. 2011). This behavior is illustrated by the WD-driven increase in dA_N/dg_s in C_3 crops, which was negatively correlated with B_t (Fig. 6A). However, this negative correlation turned into positive when considering the effects of HT in plants under WD (Fig. 6B). These interactive effects of WD and HT suggests that, under combined stress conditions, screening for rice and wheat plants with higher water use efficiency would allow the selection of plants with higher growth capacity. Although results from the present study allow relating plant growth to leaf water use efficiency, alterations in the harvest index have been reported under high temperature and water deficit (Prasad et al. 2011) and, therefore, should be considered to evaluate the agronomic water use efficiency based on the grain yield.

Adjustments in leaf carbon balance and its underlying parameters are predictive of treatments-induced decreases in plant growth

For the studied crops, the capacity for thermal homeostasis of the instantaneous mitochondrial respiration rates measured at predawn (iR) was much lower than that of iA_N (Fig. 2A, E), in line with previous reports (Loveys et al. 2002, Silim et al. 2010).

However, this observation is by no means universal and other studies reported higher thermal acclimation potential for mitochondrial respiration than for photosynthetic CO_2 assimilation rates (Atkin and Tjoelker 2003, Yamori et al. 2005, Campbell et al. 2007, Ow et al. 2008, Way and Sage 2008, Chi et al. 2013). Similarly, the effect of water deficit on mitochondrial respiration is still a non-resolved question, with studies showing increased, decreased or non-affected rates in water stressed plants (Flexas et al. 2005, Atkin and Macherel 2009, Gimeno et al. 2010). This lack of consensus, ascribed to species-specific differences among other factors (Galmés et al. 2007a), is supported by the present results. Thus, besides the general tendency to increase iR in all three species under stress, differences were significant neither in rice and maize under CT-WD, nor in rice and wheat under HT-WD, as compared to CT-WW (Fig. 2E). Overall, in rice and maize iR was less responsive to the applied stresses, whereas wheat presented the highest increase in iR when grown under water deficit or high temperature treatments.

In all three species, the response of the night-integrated rates of mitochondrial respiration (dR) to HT-WW was different to the above described response of iR. This divergence was attributed to changes in the night-time course of respiration rates in a species dependent manner. For instance, there was a trend for a decreased rate of mitochondrial respiration during the night in HT-WW maize ($R^2 = 0.77$ and $P < 0.001$ for the relationship between mitochondrial respiration rate and nigh-time). Most existing models of plant biomass production rely on instantaneous measurements of mitochondrial respiration (Amthor 2010, Boote et al. 2013, Smith and Dukes 2013), and the ratio between mitochondrial respiration and photosynthetic CO_2 assimilation rates based on instantaneous measurements has frequently been related to plant carbon balance (e.g., Atkin et al. 2007, Campbell et al. 2007). However, the present results indicate that iR may not be representative for respiratory CO_2 losses of dark adapted leaves, particularly in HT stressed plants. This information should be taken into account to increase the accuracy of current models of biomass production aimed to assess the impacts of abiotic stresses on the leaf and whole plant carbon balance.

There are few exceptions in the literature demonstrating that thermal acclimation of mitochondrial respiration is affected by the interactive effects of growth temperature with other limiting factors or resources, such as irradiance (Zaragoza-Castells et al. 2007) and nitrogen (Archontoulis et al. 2012). In the present study thermal acclimation of the mitochondrial respiration was sensitive to irrigation in all

three species (Fig. 2). In this regard, the scarce data available up to date seems contradictory: temperature response of iR in *Fagus sylvatica* occurred irrespective of changes in soil moisture (Rodríguez-Calcerrada et al. 2010), while changes in water supply notoriously decreased the respiratory response to temperature in *Geum urbanum* (Slot et al. 2008) and *Eucalyptus saligna* (Ayub et al. 2011, Crous et al. 2011).

The limitation in plant growth due to the imposed HT and WD may result from alterations in the capacity of the photosynthetic organs to provide photoassimilates and in the whole plant respiratory demands (Smith and Dukes 2013, Zhao et al. 2013). While measuring gas exchange at the whole plant is technically complex for several reasons (Alterio et al. 2006, Pérez-Priego et al. 2010), monitoring CO_2 and O_2 fluxes in leaves is currently a routine and might be tested as an indicator of plant growth capacity. In this sense, the relationship between B_t and iR or dR was not significant for any of the species when plotting data from all treatments together ($P > 0.05$; Table S1). On the contrary, B_t correlated positively with both iA_N and dA_N, with higher correlation coefficient for dA_N in all three species (Table S1). However, the highest correlation coefficients for the three species were obtained when relating B_t with the leaf carbon balance (LCB), calculated as the integrated value of the leaf net CO_2 assimilation rate over 24 h period (Table S1 and Fig. 3). In a study assessing the effect of growth temperature in wheat and rice cultivars, Kurimoto et al. (2004) found that temperature-mediated changes in the net CO_2 assimilation rate played a prominent role in determining the variations in RGR. This trend, observed when including data from all treatments together, was further corroborated when analyzing individual treatments (Table 2). Hence, the growth response coefficient for leaf carbon balance (GRC_{LCB}) was higher than GRC_{LAR}, and the significance levels of the relationship B_t *vs*. LCB were higher or equal to those of the relationship B_t *vs*. LAR (Table 2). Overall, these results indicate that: i) the decrease in plant growth under different treatments results from adjustments in plant morphology and particularly in leaf physiology, and ii) the daily balance between photosynthesis and respiration measured on individual mature leaves is explicative of the whole plant growth capacity in plants long-term exposed to water and high temperature stress.

The capacity of crops to acclimate to individual and combined WD and HT depends on their native climatic origin

High temperature and water deficit often occur simultaneously in the field, but little is known about their combined effects on plant growth, development and physiology (Vile et al. 2012). In the present study, some traits were specific of the response to either WD or HT depending on the species (Table S2). Judging from the lack of significant interaction between water regime and temperature at the multivariate level for most of the parameters analyzed, the effects of combined irrigation and heat stress were generally additive in rice and maize (Table S2). This finding is in agreement with the results observed in different accessions of *Arabidopis* exposed to WD and HT, and suggests general independency between the mechanisms involved in the responses to these stresses (Vile et al. 2012). In wheat, by contrast, seven of the ten parameters tested showed interactive, instead of independent component of combined WD and HT (Table S2).

According to the relative affectation index (RAI) calculated for each species, wheat was the most sensitive crop to high temperature (Table 3), in agreement with previous reports describing a better adaptation for rice than for wheat (Nagai and Makino 2009). The fact that wheat ancestors were originated in cold-temperate environments could explain its higher heat sensitivity when compared to rice and maize, which were domesticated in warm-subtropical habitats (Nagai and Makino 2009, Yamori et al. 2010). Unexpectedly, rice was not the most affected species by limited irrigation, at least for the parameters selected in Table S2 (Table 3). This is in accordance with no appreciable effects of WD on stress indicator parameters such as F_v/F_m and leaf RWC in rice (Table. 1), and could be attributed to long-term effects of the stress (Panković et al. 1999) and/or the cultivars used in this study. Differences in the response to temperature and drought stress have been reported among different cultivars of wheat and rice (Kurimoto et al. 2004, Yamori et al. 2009, Prasad et al. 2011, Dahal et al. 2012). Therefore, cultivars of rice, wheat and maize adapted to contrasting environmental conditions should be surveyed to confirm the trends observed in the present study.

Table S2. Significance test of irrigation, temperature and irrigation × temperature factors for maximum quantum yield of PSII at pre-dawn (F_v/F_m), relative water content (RWC), mid-morning instantaneous stomatal conductance (ig_s), total plant biomass (B_t), leaf area ratio (LAR), instantaneous net CO_2 assimilation rate (iA_N), instantaneous mitochondrial respiration at pre-dawn (iR), leaf carbon balance (LCB), instantaneous intrinsic water use efficiency (iA_N/ig_s), and leaf carbon isotope discrimination ($\delta^{13}C$). The significance level was considered * when $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. n.s.: non-significant ($P > 0.05$).

Rice										
	F_v/F_m	RWC	B_t	LAR	iA_N	ig_s	iR	LCB	iA_N/ig_s	$\delta^{13}C$
Irrigation	n.s.	n.s.	**	n.s.	***	***	n.s.	***	***	n.s.
Temperature	n.s.	n.s.	***	***	n.s.	*	n.s.	***	***	***
Irrigation × temperature	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	**	n.s.
Wheat										
	F_v/F_m	RWC	B_t	LAR	iA_N	ig_s	iR	LCB	iA_N/ig_s	$\delta^{13}C$
Irrigation	n.s.	**	***	*	***	***	**	***	***	***
Temperature	***	n.s.	***	***	**	n.s.	***	***	***	***
Irrigation × temperature	**	n.s.	**	*	n.s.	**	***	***	***	n.s.
Maize										
	F_v/F_m	RWC	B_t	LAR	iA_N	ig_s	iR	LCB	iA_N/ig_s	$\delta^{13}C$
Irrigation	**	***	***	***	**	**	n.s.	***	**	***
Temperature	*	**	***	**	n.s.	n.s.	**	n.s.	n.s.	n.s.
Irrigation × temperature	**	n.s.	***	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.

Table 3. Relative affectation index (RAI) calculated for each species by dividing the sum of significance level for each parameter by the potential maximum significance under each stress, according to Table S2.

	Rice	Wheat	Maize
Irrigation	0.5	0.8	0.8
Temperature	0.5	0.8	0.3
Irrigation × temperature	0.1	0.5	0.2

Concluding remarks

With the aim to deepen into the detrimental effects of global climate change on the three main crops worldwide – rice, wheat and maize –, the present study compares the effects of moderate drought and heat stresses on plant growth and relates these effects with adjustments in a number of morphological and physiological plant traits. Different from most examples in literature, the individual stress effects were compared with those produced by the combination of both stresses. Moreover, measurements were taken on plants long-term grown under stress and thus, testing the acclimation response rather than short-term adjustments.

A large number of morphological and physiological parameters were affected under the different treatments, in a species- and stress-specific manner. Among these parameters, daily integrated leaf CO_2 fluxes – or the leaf carbon balance –, rather than instantaneous measurements, were highly explicative of changes in plant biomass accumulation under stress in the three species. For the particular cultivars included in the study, the results showed that the effects of simultaneous HT and WD on plant traits follow mostly interactive trends in wheat and additive trends in rice and maize. These findings are relevant for modelling plant growth under stress in their need to incorporate: i) daily integrated leaf gas-exchange measurements, and ii) complex interactions of stresses that typically co-occur in the field environments.

Author contributions

JAP and JG designed the research; JAP performed the growth and gas exchange analyses; MRC measured the leaf carbon isotopic composition. All authors analysed the data and wrote the paper.

Acknowledgements

The study was financially supported by the Spanish Ministry of Science and Innovation through project AGL2009-07999 (Plan Nacional, Spain) awarded to JG. JAP was recipient of a pre-doctoral fellowship of Conselleria d'Educació, Cultura i Universitats (Govern de les Illes Balears). We are grateful to Biel Martorell (SCT-UIB) for helping in the analysis of ^{13}C.

References

Ainsworth EA, Ort DR (2010) How do we improve crop production in a warming world? Plant Physiol 154: 526–530

Alterio G, Giorio P, Sorrentino G (2006) Open-system chamber for measurements of gas exchanges at plant level. Environ Sci Technol 40: 1950–1955

Amthor JS (2010) From sunlight to phytomass: on the potential efficiency of converting solar radiation to phyto-energy. New Phytol 188: 939–959

Aranjuelo I, Irigoyen J, Nogues S, Sanchez-diaz M (2009) Elevated CO_2 and water-availability effect on gas exchange and nodule development in N_2-fixing alfalfa plants. Environ Exp Bot 65: 18–26

Archontoulis SV, Yin X, Vos J, Danalatos NG, Struik PC (2012) Leaf photosynthesis and respiration of three bioenergy crops in relation to temperature and leaf nitrogen: how conserved are biochemical model parameters among crop species? J Exp Bot 63: 895–911

Atkin OK, Scheurwater I, Pons T (2007) Respiration as a percentage of daily photosynthesis in whole plants is homeostatic at moderate, but not high, growth temperatures. New Phytol 174: 367–380

Atkin OK, Loveys BR, Atkinson LJ, Pons TL (2006) Phenotypic plasticity and growth temperature: understanding interspecific variability. J Exp Bot 57: 267–281

Atkin OK, Macherel D (2009) The crucial role of plant mitochondria in orchestrating drought tolerance. Ann Bot 103: 581–597

Atkin OK, Tjoelker MG (2003) Thermal acclimation and the dynamic response of plant respiration to temperature. Trends Plant Sci 8: 343–351

Ayub G, Smith RA, Tissue DT, Atkin OK (2011) Impacts of drought on leaf respiration in darkness and light in Eucalyptus saligna exposed to industrial-age atmospheric CO_2 and growth temperature. New Phytol 190: 1003–1018

Berry J, Björkman O (1980) Photosynthetic response and adaptation to temperature in higher-plants. Physiol Plant Mol Biol 31: 491–543

Boote KJ, Jones JW, White JW, Asseng S, Lizaso JI (2013) Putting mechanisms into crop production models. Plant Cell Environ 36: 1658–1672

Campbell C, Atkinson L, Zaragoza-Castells J, Lundmark M, Atkin OK, Hurry V (2007) Acclimation of photosynthesis and respiration is asynchronous in response to changes in temperature regardless of plant functional group. New Phytol 176: 375–389

Carmo-Silva AE, Gore MA, Andrade-Sanchez P, French AN, Hunsaker DJ, Salvucci ME (2012) Decreased CO_2 availability and inactivation of Rubisco limit photosynthesis in cotton plants under heat and drought stress in the field. Environ Exp Bot 83: 1–11

Centritto M, Lauteri M, Monteverdi MC, Serraj R (2009) Leaf gas exchange, carbon isotope discrimination, and grain yield in contrasting rice genotypes subjected to water deficits during the reproductive stage. J Exp Bot 60: 2325–2339

Chi Y, Xu M, Shen R, Wan S (2013) Acclimation of leaf dark respiration to nocturnal and diurnal warming in a semiarid temperate steppe. Funct Plant Biol 40: 1159–1167

Crafts-Brandner SJ, Salvucci ME (2000) Rubisco activase constrains the photosynthetic potential of leaves at high temperature and CO_2. Proc Natl Acad Sci USA 97: 13430–13435

Crafts-brandner SJ, Salvucci ME (2002) Sensitivity of photosynthesis in a C_4 plant, Maize, to heat stress. Plant Physiol 129: 1773–1780

Crawford A, McLachlan D, Hetherington A, Franklin K (2012) High temperature exposure increases plant cooling capacity. Curr Biol 22: 396–397

Crous KY, Zaragoza-Castells J, Löw M, Ellsworth DS, Tissue DT, Tjoelker MG, Barton CVM, Gimeno TE, Atkin OK (2011) Seasonal acclimation of leaf respiration in Eucalyptus saligna trees: impacts of elevated atmospheric CO_2 and summer drought. Glob Chang Biol 17: 1560–1576

Ehdaie B, Hall A, Farquhar GD, Nguyen H, Waines J (1991) Water-use efficiency and carbon isotope discrimination in wheat. Crop Sci 31: 1282–1288

Escalona JM, Tomàs M, Martorell S, Medrano H, Ribas-Carbo M, Flexas J (2012) Carbon balance in grapevines under different soil water supply: importance of whole plant respiration. Aust J Grape Wine Res 18: 308–318

Evans JR, von Caemmerer S (2013) Temperature response of carbon isotope discrimination and mesophyll conductance in tobacco. Plant Cell Environ 36: 745–756

FAO (2010) The state of food and Agriculture. Available at http://www.fao.org/docrep/013/i2050e/i2050e.pdf (accessed 10 May 2014)

Farquhar GD, Ehleringer J, Hubick K (1989) Carbon isotope discrimination and photosynthesis. Annu Rev plant Physiol plant Mol Biol 40: 503–537

Farquhar GD, Richards RA (1984) Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. Aust J Plant Physiol 11: 539–552

Flexas J, Barbour MM, Brendel O, Cabrera HM, Carriquí M, Díaz-Espejo A, Douthe C, Dreyer E, Ferrio JP, Gago J, Gallé A, Galmés J, Kodama N, Medrano H, Niinemets Ü, Peguero-Pina JJ, Pou A, Ribas-Carbó M, Tomàs M, Tosens T, Warren CR (2012) Mesophyll diffusion conductance to CO_2: an unappreciated central player in photosynthesis. Plant Sci 193-194: 70–84

Flexas J, Barón M, Bota J, Ducruet JM, Gallé A, Galmés J, Jiménez M, Pou A, Ribas-Carbó M, Sajnani C, Tomàs M, Medrano H (2009) Photosynthesis limitations during water stress acclimation and recovery in the drought-adapted *Vitis* hybrid Richter-110 (*V. berlandierixV. rupestris*). J Exp Bot 60: 2361–2377

Flexas J, Bota J, Galmes J, Medrano H, Ribas-Carbó M (2006) Keeping a positive carbon balance under adverse conditions: responses of photosynthesis and respiration to water stress. Physiol Plant 127: 343–352

Flexas J, Galmés J, Gallé A, Gulías J, Pou A, Ribas-Carbó M, Tomàs M, Medrano H (2010) Improving water use efficiency in grapevines: potential physiological targets for biotechnological improvement. Aust J Grape Wine Res 16: 106–121

Flexas J, Galmés J, Ribas-Carbó M, Medrano H (2005) The effects of water stress on plant respiration. In: Lambers H, Ribas-Carbó M (eds) Advances in Photosynthesis and Respiration. Plant Respiration: From Cell to Ecosystem. Kluwer Academic, Dordrecht, pp 85–94

Flexas J, Medrano H (2002) Drought-inhibition of photosynthesis in C_3 plants: stomatal and non stomatal limitations revisited. Ann Bot 89: 183–189

Flexas J, Niinemets Ü, Gallé A, Barbour MM, Centritto M, Diaz-Espejo A, Douthe C, Galmés J, Ribas-Carbo M, Rodriguez PL, Rosselló F, Soolanayakanahally R, Tomás M, Wright IJ, Farquhar GD, Medrano H (2013) Diffusional conductances to CO_2 as a target for increasing photosynthesis and photosynthetic water-use efficiency. Photosynth Res 117: 45–59

Foresight (2011) The future of food and farming: challenges and choices for global sustainability. Final Project Report, The Government Office for Science (in London)

Galmés J, Aranjuelo I, Medrano H, Flexas J (2013) Variation in Rubisco content and activity under variable climatic factors. Photosynth Res 117: 73-90

Galmés J, Cifre J, Medrano H, Flexas J (2005) Modulation of relative growth rate and its components by water stress in Mediterranean species with different growth forms. Oecologia 145: 21–31

Galmés J, Conesa MÀ, Ochogavía JM, Perdomo JA, Francis DM, Ribas-Carbó M, Savé R, Flexas J, Medrano H, Cifre J (2011) Physiological and morphological adaptations in relation to water use efficiency in Mediterranean accessions of *Solanum lycopersicum*. Plant Cell Environ 34: 245–260

Galmés J, Flexas J, Medrano H, Niinemets Ü, Valladares F (2012) Ecophysiology of photosynthesis in semi-arid environments. In: Flexas J, Loreto F, Medrano H (eds) Terrestrial Photosynthesis in a Changing Environment. Cambridge University Press, Cambridge, pp 453–469

Galmés J, Flexas J, Savé R, Medrano H (2007c) Water relations and stomatal characteristics of Mediterranean plants with different growth forms and leaf habits: responses to water stress and recovery. Plant Soil 290: 139–155

Galmés J, Pou A, Alsina M, Tomàs M, Medrano H, Flexas J (2007b) Aquaporin expression in response to different water stress intensities and recovery in Richter-110 (*Vitis* sp.): relationship with ecophysiological status. Planta 226: 671–681

Galmés J, Ribas-Carbó M, Medrano H, Flexas J (2007a) Response of leaf respiration to water stress in Mediterranean species with different growth forms. J Arid Environ 68: 206–222

Gimeno T, Sommerville K, Valladares F, Atkin OK (2010) Homeostasis of respiration under drought and its important consequences for foliar carbon balance in a drier climate: insights from two contrasting *Acacia* species. Funct Plant Biol 37: 323–333

Gratani L, Varone L, Catoni R (2008) Relationship between net photosynthesis and leaf respiration in Mediterranean evergreen species. Photosynthetica 46: 567–573

Hikosaka K, Ishikawa K, Borjigidai A, Muller O, Onoda Y (2006) Temperature acclimation of photosynthesis: mechanisms involved in the changes in temperature dependence of photosynthetic rate. J Exp Bot 57: 291–302

Hu L, Wang Z, Huang B (2010) Diffusion limitations and metabolic factors associated with inhibition and recovery of photosynthesis from drought stress in a C_3 perennial grass species. Physiol Plant 139: 93–106

Keys AJ (1999) Biochemestry of photorespiration and the consequences for plant performance. In: Bryant JA, Burrell MM, Kruger NJ (eds) Plant carbohydrate biochemestry. BIOS Scientific Publishers, Oxford, pp 147–162

Konings H (1989) Physiological and morphological differences between plants with a high NAR or a high LAR as related to environmental conditions. In: Lambers H, Cambridge M, Konings H, Pons T (eds) Causes and Consequences of Variation in Growth Rate and Productivity of Higher Plants. SPB Academic Publishing, The Hague, pp 101–123

Kubien DS, Sage RF (2008) The temperature response of photosynthesis in tobacco with reduced amounts of Rubisco. Plant Cell Environ 31: 407–418

Kurimoto K, Day D, Noguchi K (2004) Effect of respiratory homeostasis on plant growth in cultivars of wheat and rice. Plant Cell Environ 27: 853–862

Lambers H, Freijsen N, Poorter H, Hirose T, Van der Werf A (1989) Analyses of growth based on net assimilation rate and nitrogen productivity: their physiological background. In: Lambers H, Cambridge M, Konings H, Pons T (eds) Causes and Consequences of Variation in Growth Rate and Productivity of Higher Plants. SPB Academic Publishing, The Hague, pp 101–123

Li Y-F, Wang Y, Tang Y, Kakani VG, Mahalingam R (2013) Transcriptome analysis of heat stress response in switchgrass (*Panicum virgatum* L.). BMC Plant Biol 13: 153

Lobell DB, Burke MB, Tebaldi C, Mastrandrea MD, Falcon WP, Naylor RL (2008) Prioritizing climate change adaptation needs for food security in 2030. Science 319: 607–610

Long SP, Ort DR (2010) More than taking the heat: crops and global change. Curr Opin Plant Biol 13: 241–248

Loveys BR, Atkinson LJ, Sherlock DJ, Roberts RL, Fitter AH, Atkin OK (2003) Thermal acclimation of leaf and root respiration: an investigation comparing inherently fast- and slow-growing plant species. Glob Chang Biol 9: 895–910

Loveys BR, Scheurwater I, Pons TL, Fitter AH, Atkin OK (2002) Growth temperature influences the underlying components of relative growth rate: an investigation using inherently fast- and slow-growing plant species. Plant Cell Environ 25: 975–988

McCree K (1986) Whole-plant carbon balance during osmotic adjustment to drought and salinity stress. Aust J Plant Physiol 13: 33–46

Mittler R (2006) Abiotic stress, the field environment and stress combination. Trends Plant Sci 11: 15-19

Nagai T, Makino A (2009) Differences between rice and wheat in temperature responses of photosynthesis and plant growth. Plant Cell Physiol 50: 744–55

Nelson G, Mark WR, Koo J, Robertson R, Sulser T, Zhu T, Ringler C, Msangi S, Palazzo A, Batka M, Magalhaes M, Valmonte-santos R, Ewing M, Lee D (2009) Climate change: Impact on agriculture and costs of adaptation. International Food Policy Research Institute, Washington

Ow LF, Griffin KL, Whitehead D, Walcroft AS, Turnbull MH (2008) Thermal acclimation of leaf respiration but not photosynthesis in *Populus deltoides x nigra*. New Phytol 178: 123–134

Parry MAJ, Andralojc PJ, Khan S, Lea PJ, Keys AJ (2002) Rubisco activity: Effects of drought stress. Ann Bot 89: 833–839

Panković D, Sakac Z, Kevresan S, Plesnicar M (1999) Acclimation to long-term water deficit in the leaves of two sunflower hybrids: photosynthesis, electron transport and carbon metabolism. J Exp Bot 50: 127–138

Pérez P, Alonso A, Zita G, Morcuende R, Martínez-Carrasco R (2011) Down-regulation of Rubisco activity under combined increases of CO_2 and temperature minimized by changes in Rubisco k_{cat} in wheat. Plant Growth Regul 65: 439–447

Pérez-Priego O, Testi L, Orgaz F, Villalobos J (2010) A large closed canopy chamber for measuring CO_2 and water vapour exchange of whole trees. Environ Exp Bot 68: 131–138

Pinheiro C, Chaves MM (2011) Photosynthesis and drought: can we make metabolic connections from available data? J Exp Bot 62: 869–82

Poorter H, Nagel O (2000) The role of biomass allocation in the growth response of plants to different levels of light, CO_2, nutrients and water: a quantitative review. Aust J Plant Physiol 27: 595–607

Poorter H, Niinemets Ü, Poorter L, Wright IJ, Villar R (2009) Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. New Phytol 182: 565–588

Poorter H, Niinemets U, Walter A, Fiorani F, Schurr U (2010) A method to construct dose-response curves for a wide range of environmental factors and plant traits by means of a meta-analysis of phenotypic data. J Exp Bot 61: 2043–2055

Poorter H, Van der Werf A (1998) Is inherent variation in RGR determined by LAR at low irradiance and by NAR at high irradiance? A review of herbaceous species. In: Lambers H, Poorter H, Van Vuuren M (eds) Inherent Variation in Plant Growth: Physiological Mechanisms and Ecological Consequences. Backhuys Publishers, Leiden, pp 309–336

Prasad PVV, Pisipati SR, Momčilović I, Ristic Z (2011) Independent and combined effects of high temperature and drought stress during grain filling on plant yield and chloroplast EF-Tu expression in spring wheat. J Agron Crop Sci 197: 430–441

Rizhsky L, Liang H, Shuman J, Shulaev V, Davletova S, Mittler R (2004) When defense pathways collide. The response of Arabidopsis to a combination of drought and heat stress. Plant Physiol 134: 1683–1696

Rodríguez-Calcerrada J, Atkin OK, Robson TM, Zaragoza-Castells J, Gil L, Aranda I (2010) Thermal acclimation of leaf dark respiration of beech seedlings experiencing summer drought in high and low light environments. Tree Physiol 30: 214–224

Rowlands D, Frame D, Ackerley D, Al E (2012) Broad range of 2050 warming from an observationally constrained large climate model ensemble. Nat Geosci 5: 256–260

Seibt U, Rajabi A, Griffiths H, Berry JA (2008) Carbon isotopes and water use efficiency: sense and sensitivity. Oecologia 155: 441–454

Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. J Exp Bot 58: 221–227

Silim SN, Ryan N, Kubien DS (2010) Temperature responses of photosynthesis and respiration in *Populus balsamifera* L.: acclimation versus adaptation. Photosynth Res 104: 19–30

Slot M, Zaragoza-Castells J, Atkin OK (2008) Transient shade and drought have divergent impacts on the temperature sensitivity of dark respiration in leaves of *Geum urbanum*. Funct Plant Biol 35: 1135–1146

Smith NG, Dukes JS (2013) Plant respiration and photosynthesis in global-scale models: incorporating acclimation to temperature and CO_2. Glob Chang Biol 19: 45–63

Stitt M (2013) Systems-integration of plant metabolism: means, motive and opportunity. Curr Opin Plant Biol 16: 381–388

Vile D, Pervent M, Belluau M, Vasseur F, Bresson J, Muller B, Granier C, Simonneau T (2012) Arabidopsis growth under prolonged high temperature and water deficit: independent or interactive effects? Plant Cell Environ 35: 702–718

Voss I, Sunil B, Scheibe R, Raghavendra AS (2013) Emerging concept for the role of photorespiration as an important part of abiotic stress response. Plant Biol 15: 713–722

Wahid A, Gelani S, Ashraf M, Foolad M (2007) Heat tolerance in plants: An overview. Environ Exp Bot 61: 199–223

Wang C, Guo L, Li Y, Wang Z (2012) Systematic comparison of C_3 and C_4 plants based on metabolic network analysis. BMC Syst Biol 6(Suppl 2): S9

Way DA, Sage RF (2008) Thermal acclimation of photosynthesis in black spruce [*Picea mariana* (Mill.) B.S.P.]. Plant Cell Environ 31: 1250–1262

Way DA, Yamori W (2014) Thermal acclimation of photosynthesis: on the importance of adjusting our definitions and accounting for thermal acclimation of respiration. Photosynth Res 119: 89–100

Wheeler T, von Braun J (2013) Climate change impacts on global food security. Science 341: 508–513

Wright G, Rao R, Farquhar G (1994) Water-use efficiency and carbon isotope discrimination in peanut under water deficit conditions. Crop Sci 34: 92–97

Yamori W, Hikosaka K, Way DA (2014) Temperature response of photosynthesis in C_3, C_4, and CAM plants: temperature acclimation and temperature adaptation. Photosynth Res 119: 101–117

Yamori W, Noguchi K, Hikosaka K, Terashima I (2009) Cold-tolerant crop species have greater temperature homeostasis of leaf respiration and photosynthesis than cold-sensitive species. Plant Cell Physiol 50: 203–215

Yamori W, Noguchi K, Hikosaka K, Terashima I (2010) Phenotypic plasticity in photosynthetic temperature acclimation among crop species with different cold tolerances. Plant Physiol 152: 388–399

Yamori W, Noguchi K, Terashima I (2005) Temperature acclimation of photosynthesis in spinach leaves: analyses of photosynthetic components and temperature dependencies of photosynthetic partial reactions. Plant Cell Environ 28: 536–547

Yousfi S, Serret MD, Márquez AJ, Voltas J, Araus JL (2012) Combined use of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ tracks nitrogen metabolism and genotypic adaptation of durum wheat to salinity and water deficit. New Phytol 194: 230–244

Zaragoza-Castells J, Sánchez-Gómez D, Valladares F, Hurry V, Atkin OK (2007) Does growth irradiance affect temperature dependence and thermal acclimation of leaf respiration? Insights from a Mediterranean tree with long-lived leaves. Plant Cell Environ 30: 820–833

Zhao J, Hartmann H, Trumbore S, Ziegler W, Zhang Y (2013) High temperature causes negative whole-plant carbon balance under mild drought. New Phytol 200: 330–339

Chapter 4

PHOTOSYNTHETIC LIMITATIONS UNDER THE INDIVIDUAL AND COMBINED EFFECTS OF HIGH TEMPERATURE AND WATER DEFICIT

4.1. BIOCHEMICAL AND DIFFUSIVE LIMITATIONS TO PHOTOSYNTHESIS IN RICE, WHEAT AND MAIZE GROWN AT HIGH TEMPERATURE AND UNDER WATER DEFICIT.

Juan Alejandro Perdomo[1], Elizabete Carmo-Silva[2], Carmen Hermida[1], Jaume Flexas[1], Jeroni Galmés[1†].

[1]*Research Group on Plant Biology under Mediterranean Conditions. Universitat de les Illes Balears, Ctra. de Valldemossa Km.7.5, Palma 07122, Spain.*
[2]*Plant Biology and Crop Science, Rothamsted Research, Harpenden, AL5 2JQ, UK.*

[†]**Corresponding author:** Jeroni Galmés
e-mail: jeroni.galmes@uib.cat
fax: +34 / 971 / 173184
tel: +34 / 971 / 252710

Abstract

The impact of combined effects of heat stress, increased vapor pressure deficit (VPD) and water deficit on the physiology of major crops needs to be better understood to help mitigate the expected negative consequences of climate change and heat waves on global agricultural productivity. To address this issue, rice, wheat and maize plants were grown at control temperature (CT, 25°C, VPD 1.8 kPa) or high temperature (HT, 38°C, VPD 3.5 kPa) both under well-watered (WW) or water deficit (WD) conditions. Gas-exchange measurements showed that, in general, the WD conditions affected the leaf conductances to CO_2, while growth at HT had a more marked effect on the biochemistry of photosynthesis. When combined, HT and WD had an additive effect in limiting photosynthesis. The negative impacts of the imposed treatments on the processes governing the leaf gas-exchange were dependent on the species. Hence, wheat presented a higher sensitivity to high temperature, while rice and maize showed a higher acclimation potential to high temperature. Rubisco and PEPC kinetic constants determined i*n vitro* at 25ºC and 38°C were used in the modeling of C_3 and C_4 photosynthesis. The results highlighted the need to use species- and temperature-specific values for Rubisco and PEPC kinetic constants for a precise parameterization of the photosynthetic response to changing environmental conditions in different crop species.

Key words

Acclimation, crops, drought, heat, mesophyll conductance, mitochondrial respiration, photosynthesis, Rubisco kinetics.

Introduction

Mean global air temperatures are predicted to rise on average by 0.3-0.6°C per decade over the next century, with heat waves supposing much larger temperature increases are becoming more frequent, intense and persistent (IPCC 2013). In certain geographical regions, increased annual temperatures and heat wave frequency might be accompanied by decreased precipitation, causing decreased water availability for plants. While predicted increases in the concentration of atmospheric CO_2 are positive for plant productivity (Long *et al.* 2006), in some agricultural regions these beneficial effects are likely to be offset by the negative impacts of increased temperature and water deficit (Gornall *et al.* 2010). Hence, future predicted environments will compromise agriculture and food security for the increasing world population. A more detailed understanding of the capacity of the main crops, which sustain human caloric intake, to respond and acclimate to water deficit and high temperature will help mitigate the negative impacts of climate change on plant productivity.

Decreased crop productivity under water deficit and high temperature tends to be primarily caused by limited photosynthetic carbon assimilation and mitochondrial respiration (Flexas *et al.* 2006a; Atkin, Scheurwater & Pons 2006; Ainsworth & Ort 2010). Under water deficit, stomatal conductance (g_s) decreases, minimizing water loss, with parallel decreases in the mesophyll conductance (g_m) (Flexas *et al.* 2013). As a side-effect, the capacity of the leaf to transfer CO_2 from the atmosphere to the sites of carboxylation in the chloroplast stroma decreases under drought conditions (Galmés *et al.* 2011). On the other hand, for most species, the photosynthetic machinery is robust under conditions of mild to moderate water deficit (Galmés, Medrano & Flexas 2007b). Therefore, water availability decreases CO_2 assimilation mainly through diffusive rather than metabolic limitations (Flexas *et al.* 2006b; Galmés *et al.* 2007b). Increased temperature often results in increased vapor pressure deficit, which may exacerbate even more the diffusional limitations.

Photosynthetic processes are highly temperature dependent, and temperatures moderately above the thermal optimum cause decreases in photosynthetic CO_2 uptake. Contrarily to water deficit, the negative impact of high temperature on the rate of CO_2 assimilation (A) is mostly due to biochemical limitations (Scafaro *et al.* 2011; Carmo-Silva *et al.* 2012). The thermal sensitivity of Rubisco activase results in deactivation of Rubisco catalytic sites at moderately high temperatures (Salvucci & Crafts-brandner

2004; Yamori *et al.* 2011). In addition, increases in the maximum catalytic rate of carboxylation (k_{cat}^c) with temperature are offset by decreases in the affinity of Rubisco for CO_2 (i.e., increments in the Michaelis-Menten constant for CO_2, K_c, and decrements in the specificity factor, $S_{c/o}$) and the lower CO_2/O_2 ratio in solution, which increases photorespiration (Sage & Kubien 2007). On the other hand, mitochondrial respiration (R) appears to be less affected by water availability but more affected by temperature compared to photosynthesis (Atkin & Tjoelker 2003; Galmés *et al.* 2007c; Atkin & Macherel 2009; Rodríguez-Calcerrada *et al.* 2010; Silim, Ryan & Kubien 2010).

The above described responses correspond to general trends mostly studied by applying stresses over relatively short periods. However, in nature plants face long-term exposure to water deficit and high temperature, and photosynthesis and mitochondrial respiration have shown to acclimate to both water (Walters 2005; Galmés, Medrano & Flexas 2006; Flexas *et al.* 2009) and heat stress (Berry & Björkman 1980; Yamori, Noguchi & Terashima 2005; Campbell *et al.* 2007; Sage & Kubien 2007), althoughthe acclimation capacity and mechanisms may differ between species (Hikosaka *et al.* 2006; Kattge & Knorr 2007; Dillaway & Kruger 2011; Scafaro *et al.* 2011; Cheesman & Winter 2013). Furthermore, in semi-arid climates like the Mediterranean, drought and heat stress occur simultaneously and interact with each other to influence plant functioning (Mittler 2006; Vile *et al.* 2012).

On the other hand, currently algorithms in many mechanistic models of carbon uptake and release in C_3 and C_4 leaves do not account for long-term responses to changes in the environmental conditions (e.g., von Caemmerer 2000; Pittermann & Sage 2001; Hu, Wang & Huang 2010). Further, these models normally assume invariable values and temperature response for the Rubisco kinetic parameters and g_m among the different species. Actually, the use of Rubisco kinetics and g_m values experimentally measured in *Nicotiana tabacum* has been popularized in most of the studies modelling leaf gas exchange responses to variations in the environment (Bernacchi *et al.* 2001, 2002; Bernacchi, Pimentel & Long 2003; Diaz-Espejo 2013; von Caemmerer 2013). However, it has been recently highlighted that differences among species in Rubisco kinetic constants and g_m, as well as their dependence to temperature (i) exist, (ii) induce differences in photosynthetic responses to temperature, and (iii) significantly bias modeling photosynthesis (Diaz-Espejo 2013; Martins *et al.* 2013; Walker *et al.* 2013). Further, although C_4 modelling at variable temperature has been barely tackled (Massad, Tuzet & Bethenod 2007; Sage & Kubien 2007; von Caemmerer

2013), it is important that further approaches incorporate the temperature dependence of phosphoenolpyruvate carboxylase (PEPC), and the thermal response of the underlying kinetic parameters, like the affinity of PEPC for CO_2 (K_P).

Rice (*Oryza sativa*), wheat (*Triticum aestivum*) and maize (*Zea mays*) are the three major commercially important crops, accounting for ~85% of global cereal production and contributing the majority of human calories eaten directly as staple foods or indirectly through consumption of livestock fed with grain (Grassini, Eskridge & Cassman 2013). These cereals were domesticated in different climates and differ largely in their growth environments: rice and maize are cultivated in tropical hot and wet climates, whereas wheat tends to be grown in cooler temperate climates (Makino 2011). Further, these species differ in their photosynthetic mechanism, being maize a C_4 crop, and rice and wheat C_3 crops. The objectives of the present study were: i) to analyze the patterns of response of leaf photosynthesis and respiration in these three crops to long-term drought, VPD and temperature stress; ii) to compare the sensitivity and acclimation capacity of leaf photosynthesis and respiration to these stresses of the three species; and iii) to determine the improvement in the accuracy of the C_3 and C_4 photosynthetic models after incorporation of species-specific kinetics of Rubisco and PEPC, and their species-specific response to temperature.

Material and methods

Plant material, growth conditions and treatments

Plants of rice (*Oryza sativa* L. cv. Bomba), wheat (*Triticum aestivum* L. cv. Cajeme) and maize (*Zea mays* cv. Carella) were grown from seed in a greenhouse in 3.5 L pots containing a 70:30 mixture (by vol.) of horticultural substrate (Projar S.A, Spain) and perlite (granulometry A13, Projar S.A, Spain). After 2 weeks, seedlings were selected to uniform size and were moved to a walk-in-room chamber (phytotron), under controlled conditions of light intensity, photoperiod, relative humidity and temperature. Light was provided by metal halide lamps (OSRAM, Germany) placed at specific distances from the plants to obtain a photosynthetically active photon flux density (PPFD) of 500 µmol m^{-2} s^{-1}, with a photoperiod of 12 h day/12 h night. Ambient temperature and relative humidity were monitored with portable sensors Testo 175-H1 data logger (Gerilab, Spain). Relative humidity was maintained between 40 - 60% using humidifiers. For logistical reasons, Assays were performed in two separate rounds of experiments with two plants batches of similar age. A first batch of plants of the three species were grown at the control temperature (CT, 25/20°C; VPD, 1.8/1.0 kPa day/night); and a second batch of plants were grown at high temperature (HT, 38/33ºC; VPD, 3.5/2.3 kPa day/night). Only temperature and VPD differed between batches while all other environmental conditions (e.g. light intensity and quality, air removal, photoperiod duration) were constant and computer controlled.

For each batch, i.e. for each combined temperature/VPD treatment, ten pots per species were grown at soil field capacity until plants presented fully expanded leaves (typically two weeks). Thereafter, twenty days after germination, pots of all species were randomly assigned to the two irrigation treatments: five pots per species were maintained at field capacity throughout the experiment (well-watered treatment, WW), and five were maintained at 45% of field capacity (moderate water deficit treatment, WD). The level of water availability was determined gravimetrically by weighting the pots daily and maintained by compensating water losses with 50% Hoagland's solution. New leaves were allowed to develop and expand under the two irrigation treatments for a minimum of 30 days. All measurements and samples were taken at least forty days after the water treatment was initiated (i.e., 60 days after germination) on new leaves developed completely under the temperature and/or water treatments.

Gas exchange and chlorophyll a fluorescence measurements

Leaf gas exchange and chlorophyll *a* fluorescence measurements were performed with a portable photosynthesis system (Li-6400; Li-Cor Inc., USA) equipped with a leaf chamber fluorometer (Li-6400-40, Li-Cor Inc.), the latter using the multi-flash protocol (Loriaux *et al.* 2013). The response of net CO_2 assimilation rate (A_N) to varying intercellular airspace CO_2 concentration (C_i) was measured on the youngest fully expanded leaf at a saturating photosynthetic active radiation (PAR) of 1500 µmol m^{-2} s^{-1} (10% blue light), a relative humidity of the incoming air between 40 and 50% and at two leaf temperatures: 25°C and 38°C. A_N-C_i curves were initiated by allowing the leaf to reach steady-state A_N and stomatal conductance (g_s) at a CO_2 concentration in the leaf chamber (C_a) of 400 µmol CO_2 mol^{-1} air, before varying the C_a between 50 and 2000 µmol CO_2 mol^{-1} air. Corrections for the leakage of CO_2 into and out of the leaf chamber were applied to all gas-exchange data (Flexas *et al.* 2007).

The photochemical efficiency of photosystem II (Φ_{PSII}) was determined according to Genty, Briantais & Baker (1989):

$$\Phi_{PSII} = (F_m' - F_s) / F_m' \qquad [1]$$

where F_s is the steady-state fluorescence yield and F_m' the maximum fluorescence yield obtained with a light-saturating pulse of 8000 µmol m^{-2} s^{-1}.

Φ_{PSII} was used for the calculation of the linear rate of electron transport (ETR) according to Krall & Edwards (1992):

$$ETR = \Phi_{PSII} \cdot PPFD \cdot \alpha \cdot \beta \qquad [2]$$

where α is the leaf absorbance and β is the partitioning of absorbed quanta between photosystems I and II. β was assumed to be 0.5 (Laisk & Loreto 1996; Tosens *et al.* 2012). α was measured for all species grown under each treatment inside a dark chamber using the light source from the Li-6400 and a spectroradiometer (HR2000CG-UV-NIR; Ocean Optics Inc., USA) for the range 325-1075 nm, as described by Schultz (1996). α values ranged between 0.87 for measurements at 25°C and 0.86 for measurements at 38°C, with non-significant differences between species and species × treatment combinations.

Modelling C_3 photosynthesis in wheat and rice

From combined gas-exchange and chlorophyll *a* fluorescence measurements, mesophyll conductance to CO_2 (g_m) was estimated for wheat and rice according to the variable J method (Harley *et al.* 1992):

$$g_m = A_N / (C_i - (\Gamma^* (ETR + 8 (A_N + R_L)) / (ETR - 4 (A_N + R_L)))) \quad [3]$$

where A_N and C_i were obtained from gas exchange measurements at saturating light. The rate of non-photorespiratory CO_2 evolution in the light (R_L) was determined as half of the mitochondrial respiration at pre-dawn (R_{dark}), which was measured at a C_a of 400 µmol CO_2 mol^{-1} air and leaf temperatures of 25°C or 38°C. The chloroplast CO_2 compensation point in the absence of mitochondrial respiration (Γ^*) was calculated from the *in vitro* measurements of Rubisco specificity factor ($S_{c/o}$) as:

$$\Gamma^* = \frac{0.5\,O}{S_{c/o}} \quad [4]$$

A_N-C_i curves were converted into A_N-C_c curves using the values of g_m:

$$C_c = C_i - (A_N / g_m) \quad [5]$$

Maximum velocity of Rubisco carboxylation (V_{cmax}) and maximum electron transport rate (J_{max}) were calculated from A_N-C_c curves according to Bernacchi *et al.* (2002), but using the Rubisco kinetic constants (the Michaelis-Menten constants for CO_2 and O_2 and the $S_{c/o}$) measured for each species at 25°C and 38°C. For comparative purposes, V_{cmax} and J_{max} were also calculated for rice and wheat using the values for the Rubisco kinetics parameters and respective temperature dependencies reported by Bernacchi *et al.* (2001, 2002) for tobacco.

Modelling C_4 photosynthesis in maize

The C_4 photosynthesis model described by von Caemmerer (2000) was applied to the A_N-C_i curves measured for maize as detailed by Massad *et al.* (2007), with the modifications of Carmo-Silva *et al.* (2008). The maximum velocity of Rubisco carboxylation (V_{cmax}) and the maximum velocity of PEPC carboxylation (V_{pmax}), as well as the CO_2 concentrations in the bundle sheath (C_s) and in the mesophyll cells (C_m) were estimated from the hyperbolic function describing the A_N-C_i curves using a C_i step-size of 5 µmol mol^{-1}, by applying the equations:

$$A_N = g_m(C_i - C_m) \quad [6]$$

$$A_N = \frac{C_s V_{cmax}}{C_s + K_c\left(1 + O/K_o\right)}\left(1 - \frac{\gamma^* O}{C_s}\right) - R_L \quad [7]$$

$$A_N = \frac{C_m V_{pmax}}{C_m + K_p} - g_{bs}(C_s - C_m) - R_m \quad [8]$$

In these equations, the oxygen partial pressure in the bundle sheath and mesophyll cells (O), R_L, the mesophyll mitochondrial respiration (R_m), the bundle sheath conductance to CO_2 (g_{bs}) and the mesophyll conductance to CO_2 (g_m) were assumed to be invariable between water and temperature treatments, as in Carmo-Silva *et al.* (2008) and Massad *et al.* (2007), respectively. Constant values for these parameters, which were taken from von Caemmerer (2000), were O = 210 mbar, R_L = 0.01 V_{cmax}, R_m = 0.5 R_d, g_{bs} = 3 mmol m^{-2} s^{-1} and g_m = 2 mol m^{-2} s^{-1}.

The model also requires values for kinetic constants of Rubisco and PEPC: the Rubisco specificity for CO_2/O_2 ($S_{c/o}$, from which □* is calculated as 1/0.5 $S_{c/o}$), the Michaelis-Menten constants of Rubisco for CO_2 (K_c) and O_2 (K_o), and the Michaelis-Menten constant of PEPC for CO_2 (K_p). V_{cmax} and V_{pmax} values calculated using the *in vitro* kinetic constants of maize Rubisco and PEPC at 25°C and 38°C were compared to V_{cmax} and V_{pmax} calculated using the values at 25°C for □* (0.000193), K_c (650 µbar) K_o (450 mbar) and K_p (80 µbar) reported in von Caemmerer (2000). The temperature equations provided by Bernacchi *et al.* (2001, 2002) were used to calculate values for the Rubisco kinetic constants at 38°C, while K_p was assumed to be invariable with temperature changes.

Determination of Michaelis–Menten constants of Rubisco and PEPC for their gaseous substrates

The Michaelis-Menten constants of Rubisco for CO_2 (K_c) and O_2 (K_o) were determined at 25ºC and 38ºC using leaf samples of rice, wheat and maize, by the same method as previously described (Galmés *et al.* 2014). In the present study, assays were done under either 0% O_2 (100% N_2) or 21% O_2 (in 79% N_2), and thus K_o was estimated using the formula K_c (21% O_2)=K_c (0% O_2)·(1+[O_2]/K_o).

The Michaelis-Menten constant of PEPC for CO_2 (K_p) was determined for maize at 25°C and 38°C, essentially as described by Uedan & Sugiyama (1976). PEPC was extracted from leaf samples (1.2 cm^2) by grinding in a mortar with 46 mg insoluble PVPP and 2 mL of ice-cold extraction buffer containing 50 mM Bicine-NaOH (pH 8.2), 1 mM EDTA, 0.18% (w/v) PEG4000, 11 mM ε-aminocaproic acid, 2.2 mM benzamidine, 1.8 mg bovine serum albumin (BSA), 2.8% (v/v) Tween and 1.8 mM $Na_2H_2PO_4$. The homogenate was centrifuged for 4 min at 13,000 *g* and 4°C. Eight 7 mL septum-sealed vials containing 990 µL assay buffer (50 mM Bicine-NaOH (pH 8.2), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 100 mM phosphoenolpyruvate (PEP), 20 mM

NADH, 100 mM malic dehydrogenase (MDH), 100 mM glucose-6-phospate) and varying concentrations of $NaH^{14}CO_3$ (0 to 10 mM, 1.3×10^{10} Bq mol^{-1}) were equilibrated with nitrogen (N_2) for 30 min. Reactions were started by the addition of 10 µl leaf extract, and were quenched after 1 min with 10 M formic acid.

Rubisco specificity factor determinations

Rubisco specificity for CO_2/O_2 ($S_{c/o}$) was measured at 25°C and 38°C for rice, wheat and maize (n = 6-12) using purified leaf extracts obtained as in Galmés *et al.* (2006) and the oxygen electrode (Model DW1; Hansatech, Kings Lynn., UK) method described by Parry, Keys & Gutteridge (1989). Reaction mixtures contained (final concentrations) 100 mM Bicine-NaOH (pH 8.2), 10 mM $MgCl_2$, 0.15 mg mL^{-1} carbonic anhydrase, 2 mM $NaH^{14}CO_3$ (18.5 kBq mol^{-1}), activated Rubisco from purified extracts (20 µL) and 2.5 µM RuBP. The basic buffer was pre-equilibrated with CO_2-free air at the temperature of measurement. RuBP oxygenation was calculated from the oxygen consumption and carboxylation from the amount of ^{14}C incorporated into PGA when all the RuBP had been consumed.

Temperature/VPD sensitivity and acclimation

The effect of temperature/VPD on the main leaf gas exchange parameters was examined using two indexes. The temperature sensitivity index (TSI), to assess the impact of an increase in the temperature of measurement on a given parameter (Y) in plants grown at 25°C (CT), was calculated as:

$$TSI = \frac{Y_{\mathrm{CT-25}}}{Y_{\mathrm{CT-38}}} \qquad [9]$$

The temperature acclimation index ratio (TAI) of the same leaf gas exchange parameters measured and grown at a specific temperature (Silim *et al.* 2010) was calculated as:

$$TAI = \frac{Y_{HT-38}}{Y_{CT-25}} \qquad [10]$$

Statistical analysis

The statistical significance of trait variation was tested by factorial ANOVA, with species, irrigation and temperature regimes as fixed factors, and the interaction between treatments. Post hoc comparison between treatments was performed with

Duncan test ($P < 0.05$) using Statistica 6.0 software package (StatStof Inc., USA). Regressions coefficients were calculated with the 11.0 Sigma Plot software package.

Results

Leaf CO_2 conductances and assimilation in rice, wheat and maize grown under water deficit and elevated temperature and VPD

Plants of rice, wheat and maize grown at 25°C and 1.8 kPa with optimal water supply (CT-WW) had similar values of net CO_2 assimilation rate (A_N) at 25°C (Fig. 1a). By comparison, when A_N measured at 38°C in the same plants it reached similar values than at 25ºC in maize, but was lower in rice and wheat (Fig. 1b). In plants grown at 38°C and 3.5 kPa with optimal water supply (HT-WW), A_N measured at 38°C was higher in maize than in rice or wheat, and A_N measured at 25°C was much decreased in maize, slightly decreased in rice and unchanged in wheat compared to the measurement at the higher temperature.

Growth under conditions of water deficit (CT-WD and HT-WD) had a negative impact on A_N for all plants except for maize grown at HT, mostly as a consequence of decreased stomatal conductance (g_s, Fig. 1c, d). Effects of water deficit and growth temperature on the mesophyll conductance (g_m, Fig. 1e, f), estimated for the C_3 species, showed less obvious trends. Although the comparison of the results obtained with three different methods for g_m estimation (Table S1) showed some scattering in the data, significant positive correlations ($P < 0.01$) were obtained between the method of Harley (adopted here) and two alternative methods (Ethier & Livingston 2004; Yin *et al.* 2009) (data not shown) for most of the treatments (12 out of 16). No clear pattern was observed for the 4 treatments showing discrepancies, e.g. in some cases measurements were at 25ºC and in others at 38ºC. The increase in g_m in wheat plants grown at 25°C under WD compared to WW conditions was confirmed by the three estimation methods (Table S1).

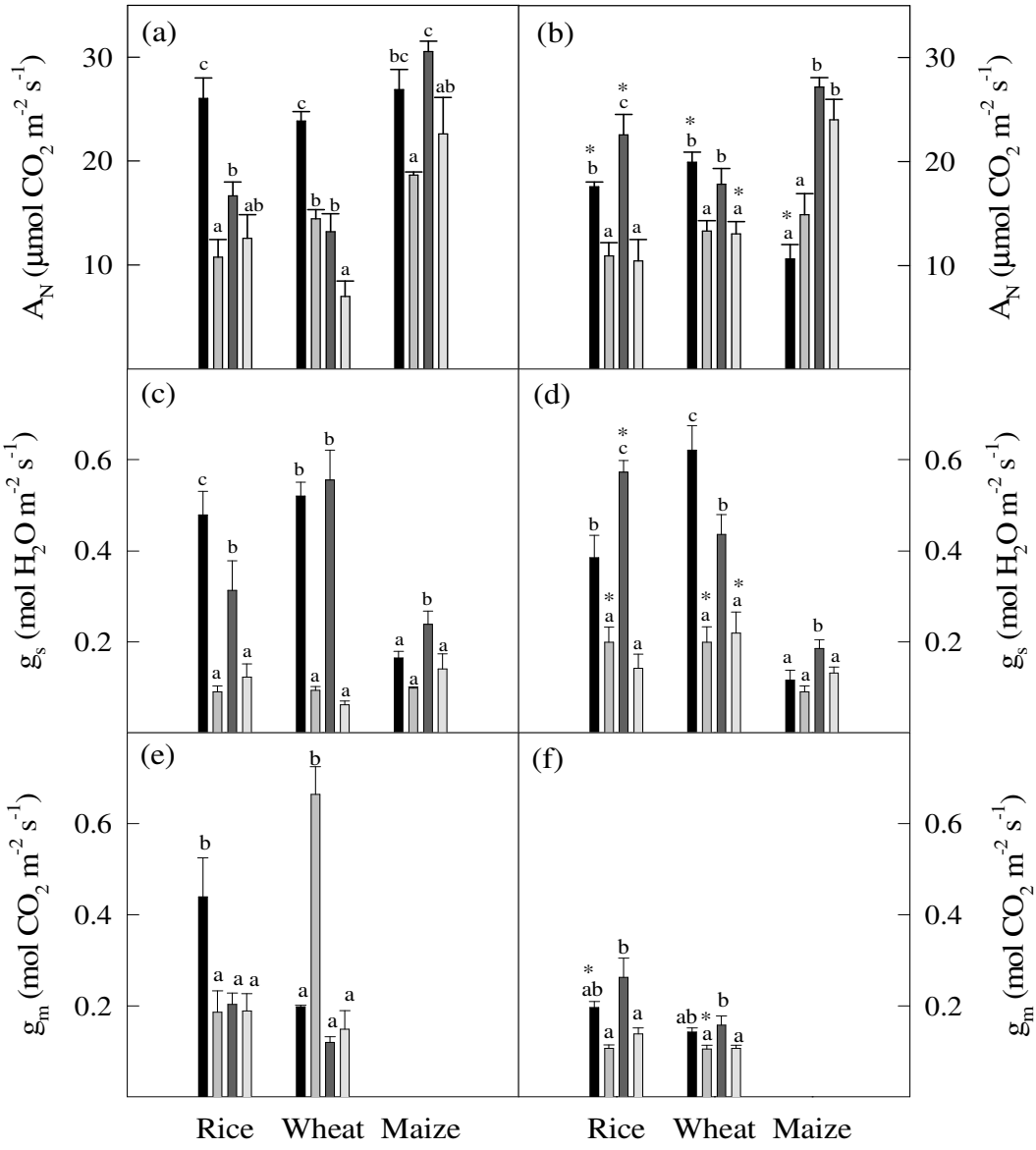

Figure 1. (a, b) The net CO_2 assimilation rate (A_N), (c, d) the stomatal conductance (g_s) and (e, f) the mesophyll conductance (g_m) in plants grown at CT (a, c, e) and HT (b, d, f) for rice, wheat and maize measured at ■ WW-25ºC, ▭ WD-25ºC, ■ WW-38ºC and ▭ WD-38ºC. Values are means ± standard error (n = 3-5). Different letters and asterisk denote statistically significant differences by Duncan analysis ($P < 0.05$) among treatments within each species and same growth temperature and between the two growth temperatures within the same species, irrigation treatment and temperature of measurement, respectively.

Table S1. Comparison of the mesophyll conductance (g_m, mol m^{-2} s^{-1}) estimated from three different methods: Harley *et al.* (1992), Ethier & Livingston (2004), Yin *et al.* (2009), in rice and wheat plants grown at CT and HT, under WW and WD conditions, and measured at 25ºC and 38ºC. The values for the Rubisco kinetic parameters required in the three methods were those measured *in vitro* in the present study for rice and wheat at the two temperatures, 25ºC and 38ºC (Table 3). Ethier method is based on gas exchange measurements, while Harley and Yin methods are based on combination of gas exchange and chlorophyll fluorescence measurements. Yin and Harley methods differ in that Yin includes the possible contributions of cyclic electron transport, pseudocyclic electron transport, and variable Q-cycle to balance H^+ and e^- supply. Differences among methods are described in more detail by Pons *et al.* (2009). Values are means ± standard errors (n = 5). Different letters denote statistically significant differences by Duncan analysis ($P < 0.05$) among the three methods within the same temperature and irrigation treatments.

Species	Growth T (ºC)	Irrigation Treatment	Measurement T (ºC)	g_m Harley's method	g_m Ethier's method	g_m Yin's method
Rice	CT	WW	25	0.439±0.086^{b}	0.335±0.027^{a}	0.456±0.091^{b}
Rice	CT	WD	25	0.187±0.046^{b}	0.123±0.020^{a}	0.102±0.026^{a}
Rice	CT	WW	38	0.204±0.025^{a}	0.192±0.032^{a}	0.172±0.032^{a}
Rice	CT	WD	38	0.189±0.038^{ab}	0.237±0.037^{b}	0.150±0.049^{a}
Rice	HT	WW	25	0.197±0.013^{a}	0.262±0.023^{b}	0.283±0.063^{b}
Rice	HT	WD	25	0.107±0.007^{a}	0.262±0.057^{b}	0.441±0.029^{c}
Rice	HT	WW	38	0.263±0.042^{a}	0.346±0.044^{b}	0.319±0.009^{b}
Rice	HT	WD	38	0.139±0.013^{a}	0.151±0.039^{a}	0.286±0.016^{b}
Wheat	CT	WW	25	0.198±0.004^{a}	0.184±0.011^{a}	0.199±0.005^{a}
Wheat	CT	WD	25	0.664±0.060^{b}	0.320±0.012^{a}	0.421±0.068^{a}
Wheat	CT	WW	38	0.121±0.012^{a}	0.097±0.013^{a}	0.089±0.017^{a}
Wheat	CT	WD	38	0.149±0.041^{b}	0.158±0.012^{b}	0.068±0.018^{a}
Wheat	HT	WW	25	0.143±0.009^{a}	0.173±0.022^{a}	0.157±0.010^{a}
Wheat	HT	WD	25	0.106±0.008^{a}	0.180±0.031^{b}	0.087±0.013^{a}
Wheat	HT	WW	38	0.158±0.020^{a}	0.308±0.023^{b}	0.116±0.024^{a}
Wheat	HT	WD	38	0.107±0.007^{b}	0.158±0.020^{c}	0.066±0.008^{a}

Decreases in g_s and g_m explained largely the limitation of A_N in rice and wheat plants under WD conditions (Fig. 1), so that a tight correlation was observed between the total leaf conductance to CO_2 (g_t, calculated from integration of g_s and g_m) and A_N (Fig. S1). A similar observation was true for g_s in maize (Fig. S1), supporting that diffusive limitations to photosynthesis are predominant in plants exposed to moderate

water deficit conditions. Conversely, in wheat plants grown at 25°C and measured at 38°C, A_N was largely decreased even though g_t was mostly unaffected (Fig. S1).

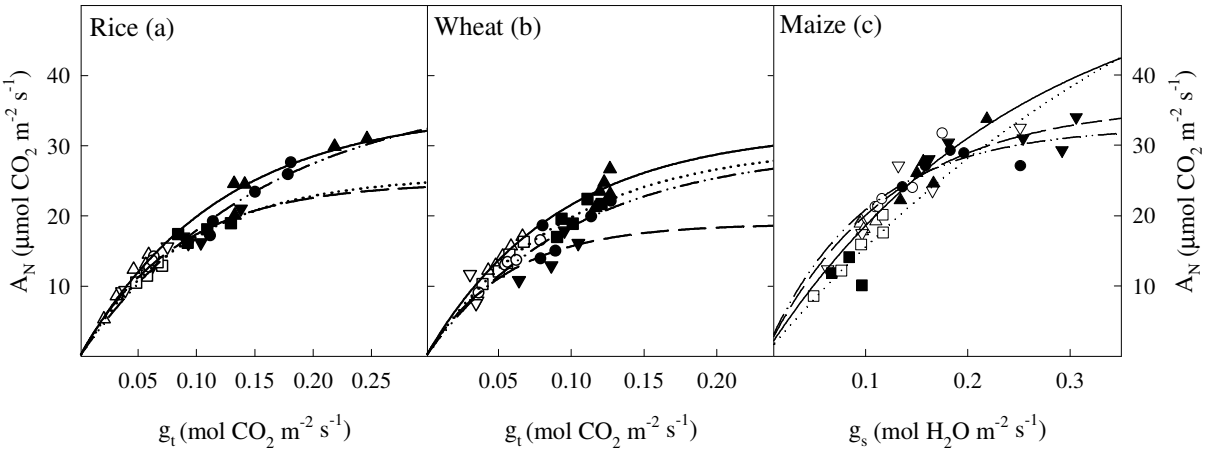

Figure S1. The relationship between the net CO_2 assimilation rate (A_N) and the total leaf conductance (g_t) (a, b) in rice and wheat, and the stomatal conductance (c) in maize. Symbols and treatments follows: ▲ CT-WW-25ºC, △ CT-WD-25ºC, ▼ CT-WW-38ºC ▽ CT-WD-38ºC, ■ HT-WW-25ºC, ■ HT-WD-25ºC, ● HT-WW-38ºC, ○ HT-WD-38ºC. Lines and treatments as follows: solid regression (——) CT-25ºC, dashed regression (- - - -) CT-38ºC, dotted regression (·····) HT-25ºC, dashed-dotted regression (— · —) HT-38ºC.

Leaf mitochondrial dark respiration (R_{dark}) in rice, wheat and maize grown under water deficit and elevated temperature and VPD

Plants of all three species grown at CT presented a similar response of the mitochondrial dark respiration rate (R_{dark}) to the imposed treatments (Fig. 2). This response consisted in a boost of R_{dark} after sudden increase in the temperature of measurement. The effects of WD on R_{dark} in CT plants were non-significant at the measurement temperature of 25°C in all three species, but became significant in wheat and maize measured at 38°C. In HT grown plants, the patterns of response of R_{dark} to the imposed treatments were radically different to those displayed by CT plants. In HT plants, R_{dark} became sensitive to the irrigation treatment in the C_3 crops (except in wheat measured at 25°C), but not in maize. On the contrary, in HT plants the effects of the temperature of measurement on R_{dark} were less evident than in CT plants, with significant changes only observed in maize and in HT-WW wheat.

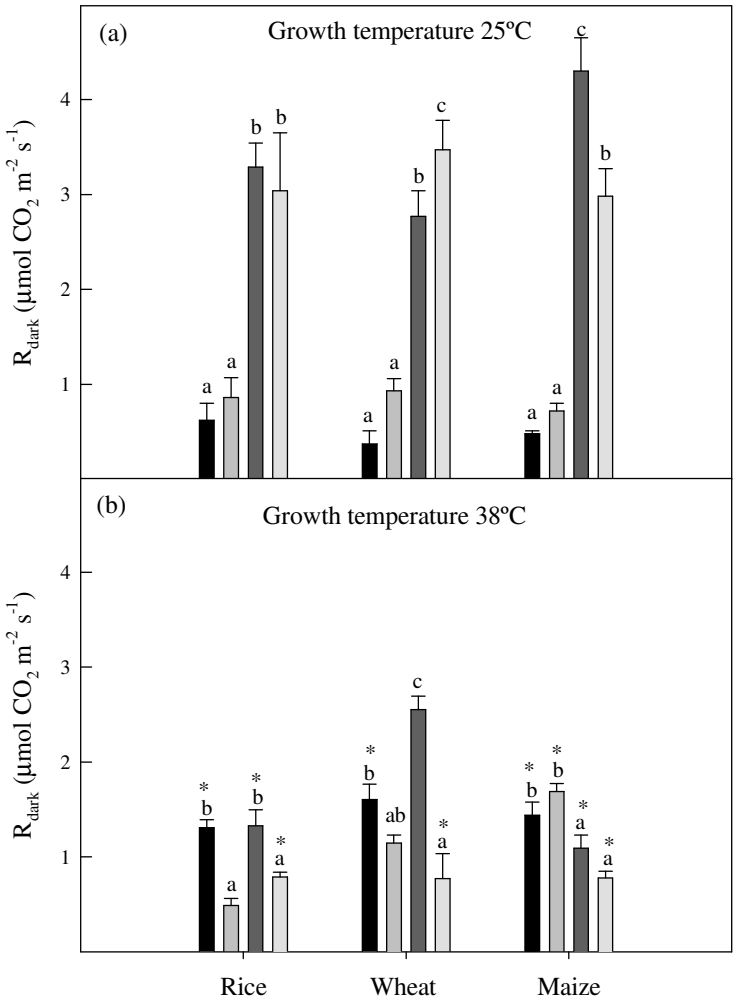

Figure 2. The mitochondrial respiration at pre-dawn (R_{dark}) in plants grown at CT (a) and HT (b) for rice, wheat and maize measured at WW-25ºC, WD-25ºC, WW-38ºC and WD-38ºC. Values are means ± standard error (n = 3-5). Different letters and asterisk denote statistically significant differences by Duncan analysis ($P < 0.05$) among treatments within each species and same growth temperature and between the two growth temperatures within the same species, irrigation treatment and temperature of measurement, respectively.

Long-term effects of water deficit and high temperature and VPD stress on the photosynthetic biochemistry of the three crops

The response of photosynthesis to increasing CO_2 concentration was analyzed in the three species on the basis of the CO_2 concentration in the chloroplastic stroma (i.e. A_N–C_c curves in rice and wheat, and A_N–C_s in maize). All crops displayed the well-described response of A_N to increasing C_c or C_s (Figs. S2, S3 and S4).

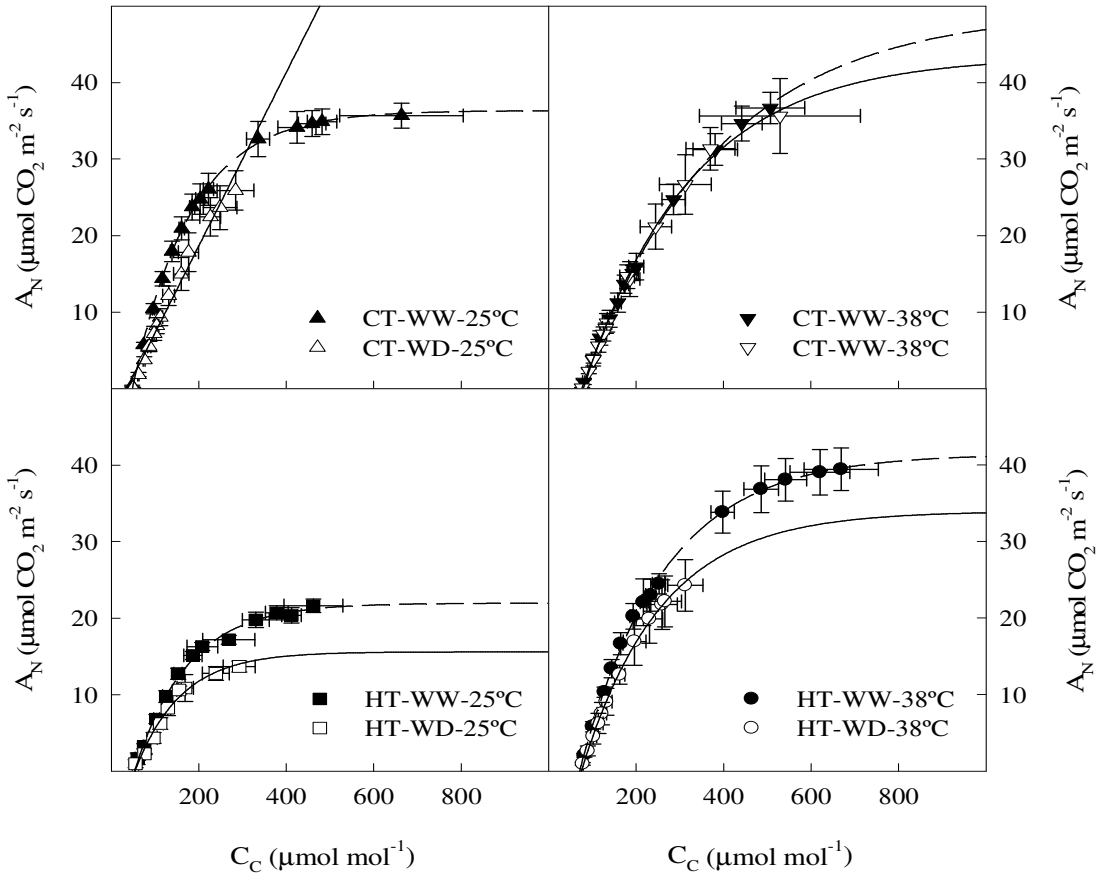

Figure S2. Relationship between the net CO_2 assimilation rate (A_N) and the chloroplastic CO_2 concentration (C_c) in rice. Values are means ± standard error (n = 5). Axis scales have been adjusted to allow comparison among panels within this figure and with Figs. S3 & S4.

In general, for rice and wheat, the effect of temperature was more evident than that of water availability on the shape of the A_N-C_c curves (Figs. S2 and S3). This observation suggests a higher resilience of the photosynthetic biochemistry to water deficit than to high temperatures. The biochemical parameters derived from A_N-C_c curves showed the same pattern. In CT plants, the maximum velocity of Rubisco carboxylation (V_{cmax}) was more responsive to the increase in temperature of measurement from 25°C to 38°C in rice than in wheat (Fig. 3a, b). By contrast, both species showed decreased V_{cmax} in HT plants when lowering the temperature of measurement from 38°C to 25°C. Significant effects of WD on V_{cmax} were observed in rice under CT-25°C and HT-38°C, and were absent in wheat.

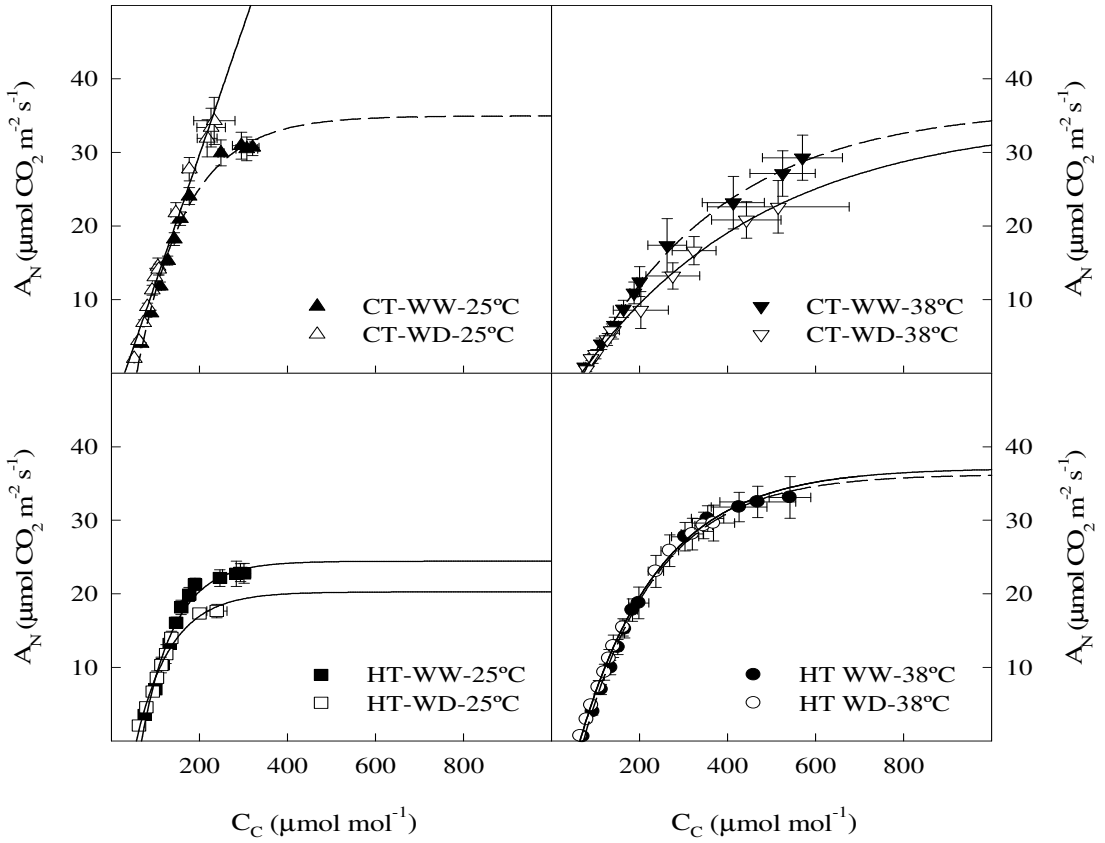

Figure S3. Relationship between the net CO_2 assimilation rate (A_N) and the chloropastic CO_2 concentration (C_c) in wheat. Values are means ± standard error (n = 5). Axis scales have been adjusted to allow comparison among panels within this figure and with Figs. S2 & S4.

Compared to V_{cmax}, the maximum rate of electron transport (J_{max}) was less affected by changes in the temperature of measurement, but similarly by changes in the irrigation treatment (Fig. 3b, c). In consequence, in both rice and wheat, the ratio J_{max}/V_{cmax} was lower when measured at 38°C compared to 25°C, irrespective of the growth temperature. The effect of the growth temperature on J_{max}/V_{cmax} ratio was significant when plants were measured at 38°C in the two species. By contrast, significant effects of WD were restricted to CT-25°C rice and CT-38°C wheat.

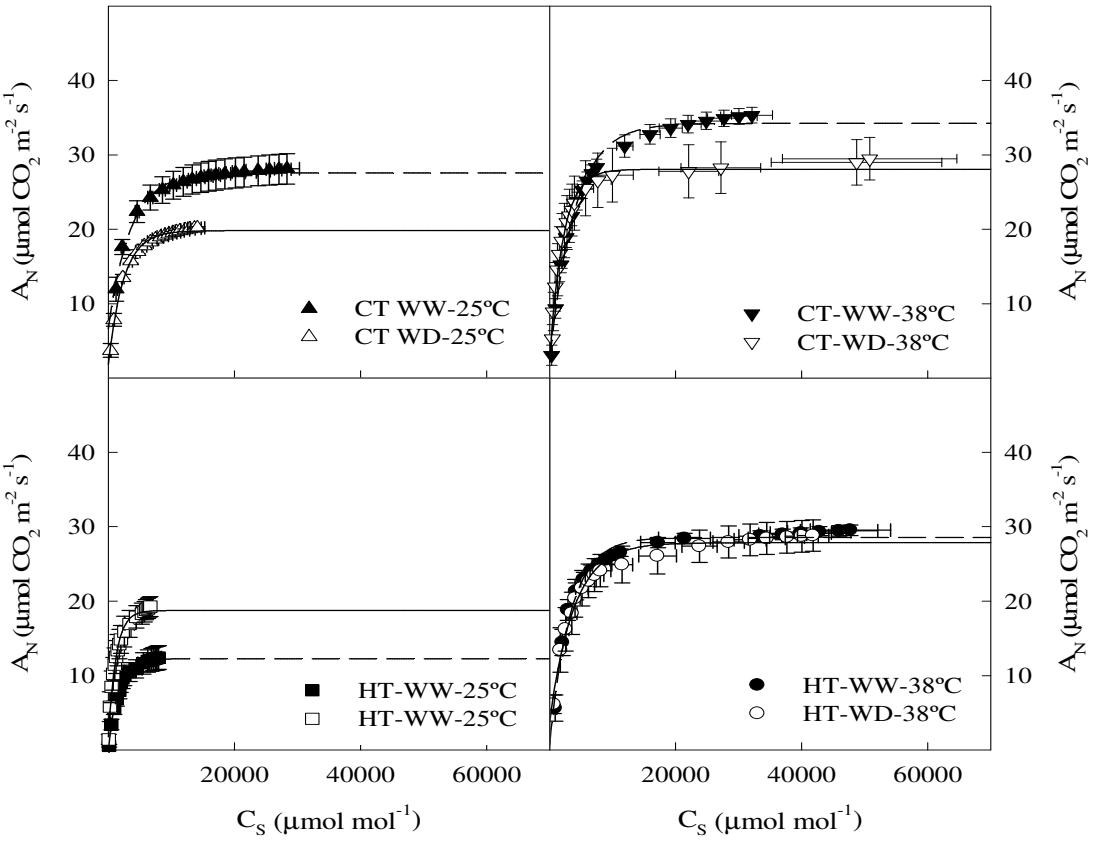

Figure S4. Relationship between the net CO_2 assimilation rate (A_N) and the bundle sheath CO_2 concentration (C_s) in maize. Values are means ± standard error (n = 5). Y-axis scale has been adjusted to allow comparison among panels within this figure and with Figs. S2 & S3.

Long-term growth under WD had more evident effects on the shape of A_N-C_s curves in maize compared to the above commented effects of the A_N-C_c curves in the C_3 crops (Fig. S3). However, these effects were restricted to the linear part of the A_N-C_s curve, informative of the PEPC activity. Accordingly, the maximum rate of PEPC carboxylation (V_{pmax}) was affected by WD under all treatments except HT-25°C (Fig. 3c, d). The effect of the temperature of measurement on V_{pmax} was dependent of the growth temperature: no effects in CT-grown plants, but dramatic decreases in HT-grown plants when decreasing the temperature of measurement from 38°C to 25°C. V_{pmax} was also highly responsive to the growth temperature, showing a high capacity for thermal acclimation (i.e., highest values when V_{pmax} was measured at the respective growth temperature). In maize, V_{cmax} was also significantly affected by the irrigation treatment in plants grown at CT, while at HT-25°C V_{cmax} increased under WD (Fig. 3a,

b). Likewise, V_{cmax} in maize increased with the temperature of measurement at both growth temperatures irrespective of water availability.

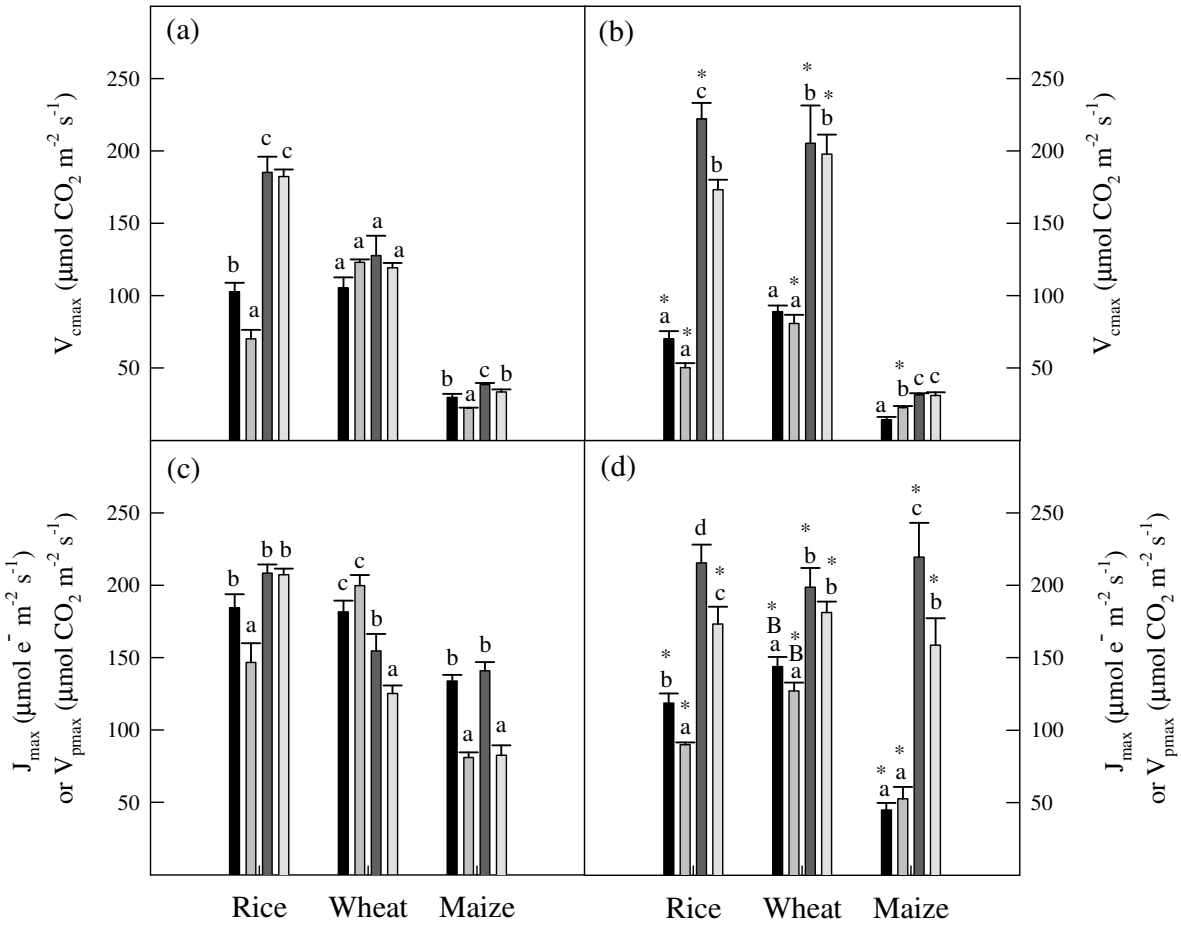

Figure 3. (a, b) The maximum velocity of Rubisco carboxylation (V_{cmax}), (c, d) the maximum electron transport rate (J_{max}) and the maximum velocity of PEPC carboxylation PEPC (V_{pmax}), in plants grown at CT (a, c) and HT (b, d). V_{cmax} were measured for wheat, rice and maize, J_{max} in wheat and rice, and V_{pmax} only in maize. All parameters were measured at ■ WW-25ºC, ▭ WD-25ºC, ■ WW-38ºC and ▭ WD-38ºC. Values are means ± standard error (n = 3-5). Different letters and asterisk denote statistically significant differences by Duncan analysis ($P < 0.05$) among treatments within each species and same growth temperature and between the two growth temperatures within the same species, irrigation treatment and temperature of measurement, respectively.

Kinetic properties of Rubisco and PEPC and their relevancy in modelling photosynthesis of C_3 and C_4 plants

The gross CO_2 assimilation rate (A_G) was calculated from the sum of A_N and half of the mitochondrial respiration in the dark (R_{dark}). In rice and wheat, A_G increased linearly with the ratio of CO_2 and O_2 concentrations in the chloroplast (C_c/O) (Fig. 4). For a given temperature treatment, WD plants showed a lower A_G due to decreased

C_c/O, in both rice and wheat. It is remarkable that rice plants measured at the same temperature but grown at different temperatures (e.g., compare CT-25°C and HT-25°C) presented different A_G values for a given C_c/O, suggesting that the carboxylase/oxygenase activity of Rubisco was sensitive to the growth temperature.

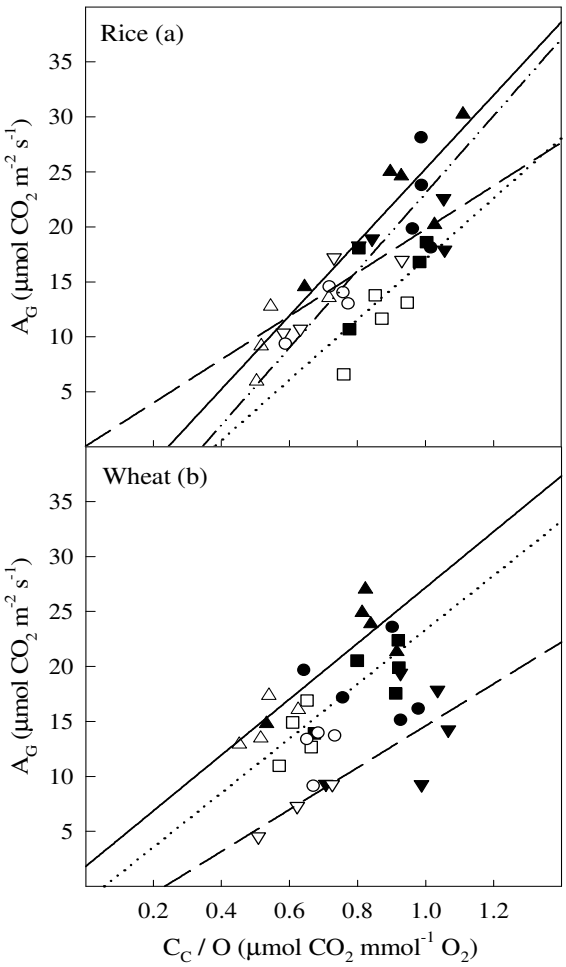

Figure 4. The relationship between the gross photosynthesis (A_G) and the relative concentrations of CO_2 and O_2 (C_c/O) for (a) rice and (b) wheat. Symbols, treatments and lines as follows: ▲ CT-WW-25ºC, △ CT-WD-25ºC, solid regression (——); ▼ CT-WW-38ºC, ▽ CT-WD-38ºC, dashed regression (- - - -); ■ HT-WW-25ºC, ■ HT-WD-25ºC, dotted regression (·····); ● HT-WW-38ºC, ○ HT-WD-38ºC, dashed-dotted regression (- · - · -). In rice, solid regression $R^2 = 0.90$ $P < 0.001$, dashed regression $R^2 = 0.69$ $P < 0.01$, dotted regression $R^2 = 0.54$ $P < 0.05$ and dashed-dotted regression $R^2 = 0.77$ $P < 0.001$. In wheat, solid regression $R^2 = 0.79$ $P < 0.001$, dashed regression $R^2 = 0.60$ $P < 0.01$ and dotted regression $R^2 = 0.65$ $P < 0.01$.

Rubisco and PEPC kinetic constants, required for photosynthesis modeling, were measured in vitro at the two temperatures of measurement to enable a more accurate modeling. As expected, all kinetic constants increased at 38°C with respect to 25°C in

the three species (Table 1). Differences between the two C_3 crops and maize were significant for the Michaelis-Menten constant of Rubisco for CO_2 (K_c) at both temperatures, and for the Michaelis-Menten constant of Rubisco for O_2 (K_o) at 38°C.

Table 1. Kinetic parameters of Rubisco and PEPC from rice, wheat and Maize measured at 25ºC and 38ºC. Four replicates were considered for the Michaelis–Menten constants of Rubisco for CO_2 and O_2 (K_c and K_o) and the Michaelis–Menten constants of PEPC for CO_2 (K_P), and five for the Rubisco specificity factor ($S_{c/o}$). The chloroplast CO_2 compensation point in the absence of mitochondrial respiration Γ*) was calculated from $S_{c/o}$ as explained in Material and Methods section. Different letters and asterisks denote statistically significant differences by Duncan analysis ($P < 0.05$) among species within the same measurement temperature and between both measurement temperatures within the same species, respectively.

Species	K_c (µmol mol^{-1})		K_o (mmol mol^{-1})		Γ*		K_P (µbar)	
	25ºC	38ºC	25ºC	38ºC	25ºC	38ºC	25ºC	38ºC
Rice	291±16^{a}	877±52^{a*}	457±46^{a}	583±57^{a*}	41.8±1.9^{a}	63.4±3.6^{b*}	-	-
Wheat	316±11^{a}	893±36^{a*}	392±38^{a}	503±45^{a*}	38.7±2.1^{a}	53.2±3.2^{a*}	-	-
Maize	858±70^{b}	1883±57^{b*}	398±35^{a}	745±75^{b*}	42.9±3.0^{a}	64.7±2.6^{b*}	80±3	132±5*

The V_{cmax} estimated for the C_3 species by applying the model of Farquhar, von Caemmerer & Berry (1980), and using the values of the *in vitro* Rubisco kinetics specific for each species at each measurement temperature/VPD (Table 1) tended to be lower than the V_{cmax} estimated using the kinetic parameters from Bernacchi *et al.* (2001, 2002) (Table 2). However, the estimates obtained by each method for the different species under each treatment were highly related. None of the differences between V_{cmax} values estimated using specific kinetics and kinetics from Bernacchi *et al.* (2001, 2002) were significant in rice, and only 3 cases were significant in wheat (CT-WD-25ºC, HT-WW-25ºC and HT-WD-38ºC). Actually, the correlation between the two V_{cmax} estimates was high ($r^2 > 0.99$) in both species, although the lower slope in wheat (0.86 compared to 0.98 in rice) made it more distant to the 1:1 relationship (data not shown).

Table 2. Comparison of the maximum velocity of Rubisco carboxylation (V_{cmax}) and maximum rate of electron transport (J_{max}) in plants grown at CT and HT, under WW and WD, and measured at 25ºC and 38ºC, using the Rubisco kinetics parameters (K_c, K_o and $S_{c/o}$) measured in the present study for rice and wheat (own kinetics), with regard to the parameters described for tobacco in Bernacchi *et al.* (2001, 2002). V_{cmax} and J_{max} were calculated on a C_c basis as estimated following the method of Harley *et al.* (1992). Values are means ± standard errors (n = 5). Asterisks denote statistically significant differences by Duncan analysis ($P < 0.05$) between the two methods within the same treatment.

Species	Growth T (ºC)	Irrigation Treatment	Measurement T (ºC)	Own kinetics		Kinetics by Bernacchi *et al.* (2001, 2002)	
				V_{cmax}	J_{max}	V_{cmax}	J_{max}
Rice	CT	WW	25	102.7±6.1	184.5±9.2	116.0±6.0	112.7±22.3*
Rice	CT	WD	25	70.1±6.2	146.7±13.2	77.9±7.2	82.8±4.5*
Rice	CT	WW	38	185.1±10.8	208.3±5.9	206.2±10.8	197.6±6.9
Rice	CT	WD	38	182.2±5.0	207.3±4.2	195.8±3.1	198.5±0.3
Rice	HT	WW	25	70.2±5.4	118.8±6.6	78.0±5.8	108.8±5.1
Rice	HT	WD	25	50.2±3.3	90.0±1.6	59.2±3.8	79.3±2.7*
Rice	HT	WW	38	222.1±11.0	215.4±12.6	228.3±14.4	198.5±11.0
Rice	HT	WD	38	173.2±6.8	173.4±11.8	176.5±15.2	161.4±5.8
Wheat	CT	WW	25	105.4±7.2	181.5±7.9	125.8±9.7	101.5±15.4*
Wheat	CT	WD	25	122.9±2.1	199.8±7.1	144.1±3.3*	81.9±2.5*
Wheat	CT	WW	38	127.7±13.7	154.7±11.6	151.6±16.2	156.9±11.3
Wheat	CT	WD	38	119.2±3.3	125.3±5.4	134.5±8.9	119.7±5.8
Wheat	HT	WW	25	88.9±4.2	143.8±6.8	110.0±6.1*	133.6±5.9
Wheat	HT	WD	25	80.9±5.9	127.2±5.7	104.5±6.1*	122.8±6.6
Wheat	HT	WW	38	205.2±26.0	198.7±13.3	247.7±29.2	186.8±10.9
Wheat	HT	WD	38	197.8±13.5	181.1±7.6	230.3±15.9	174.2±8.6

Significant differences between values of J_{max} estimated using specific and Bernacchi kinetics (Bernacchi *et al.* 2001, 2002) were observed for three treatments in rice (CT-WW-25°C, CT-WD-25°C and HT-WW-38°C) and two in wheat (CT-WW-25°C and CT-WD-25°C) (Table 2). To strengthen these observations, the estimates for V_{cmax} and J_{max} obtained using the species specific values of Rubisco kinetics measured in the present study and those reported for tobacco in Bernacchi *et al.* (2001, 2002) were compared by applying the method described by Ethier & Livingston (2004). In this case, significant differences in V_{cmax} were observed in two cases for each species, while significant differences in J_{max} were only observed in CT-WD-25°C wheat (Table S3).

Table S3. Comparison of the maximum velocity of Rubisco carboxylation (V_{cmax}) and maximum rate of electron transport (J_{max}), estimated with the method described by Ethier & Livingston (2004), in plants grown at CT and HT, under WW and WD, and measured at 25ºC and 38ºC, using the Rubisco kinetics parameters (K_c, K_o and $S_{c/o}$) measured in the present study for rice and wheat (own kinetics), with regard to the parameters described in Bernacchi *et al.* (2001, 2002). Values are means ± standard errors (n = 5). Asterisks denote statistically significant differences by Duncan analysis ($P < 0.05$) between the two methods within the same treatment.

Species	Growth T (ºC)	Irrigation Treatment	Measurement T (ºC)	Own kinetics		Kinetics by Bernacchi et al. (2001, 2002)	
				V_{cmax}	J_{max}	V_{cmax}	J_{max}
Rice	CT	WW	25	165.0±9.6	186.1±13.2	183.0±17.2	179.3±12.4
Rice	CT	WD	25	108.4±4.6	120.3±17.5	146.8±7.9*	115.1±18.4
Rice	CT	WW	38	251.2±6.2	202.7±14.1	275.7±15.2	188.9±11.1
Rice	CT	WD	38	152.8±4.8	214.1±6.6	224.2±3.2*	180.3±16.6
Rice	HT	WW	25	100.7±10.0	121.8±7.7	89.0±7.5	108.5±4.5
Rice	HT	WD	25	60.3±2.9	70.0±3.5	61.2±8.6	74.1±6.6
Rice	HT	WW	38	260.0±11.1	204.6±18.2	303.1±15.3	201.7±18.8
Rice	HT	WD	38	141.6±29.5	117.9±17.3	159.2±26.0	112.2±16.8
Wheat	CT	WW	25	173.6±10.2	182.7±10.2	169.6±3.7	170.6±4.5
Wheat	CT	WD	25	169.3±7.4	201.6±7.6	178.1±0.9	169.0±1.4*
Wheat	CT	WW	38	156.8±25.4	147.7±13.9	207.4±45.1	146.7±15.4
Wheat	CT	WD	38	99.3±21.1	123.0±19.5	150.2±22.5	124.9±9.4
Wheat	HT	WW	25	159.8±10.9	145.5±13.7	149.2±12.9	136.5±12.8
Wheat	HT	WD	25	98.2±12.5	94.3±2.8	93.0±12.3	96.1±4.5
Wheat	HT	WW	38	153.8±17.6	161.6±12.0	277.5±30.6*	169.6±14.0
Wheat	HT	WD	38	142.9±9.0	156.3±14.9	220.5±23.4*	146.7±9.6

Regarding C_4 modeling, the comparison was established between V_{cmax} and V_{pmax} estimates using the Rubisco and PEPC kinetics reported in the present study for maize and those reported in von Caemmerer (2000) (Table 3). von Caemmerer (2000) used the temperature dependence of Rubisco kinetic constants reported by Bernacchi *et al.* (2002), while K_p was assumed to be invariable with temperature. For the maize estimates, differences in V_{cmax} were non-significant under all treatments, while significant differences in V_{pmax} were found in CT-grown plants, irrespective of the irrigation treatment and the temperature of measurement (Table 3).

Table 3. Comparison of the maximum velocity of Rubisco carboxylation (V_{cmax}) and the maximum velocity of PEPC carboxylation (V_{pmax}) in plants grown at CT and HT, under WW and WD, and measured at 25ºC and 38ºC, using Rubisco and PEPCase kinetics parameters (K_c, K_o, $S_{c/o}$ and K_p) measured in the present study for Maize, with regard to the constants parameters reported by von Caemmerer (2000). Values are means ± standard errors (n = 5). Asterisks denote statistically significant differences by Duncan analysis ($P < 0.05$) between the two methods within the same treatment.

Species	Growth T (ºC)	Irrigation Treatment	Measurement T (ºC)	Own kinetics		Kinetics by von Caemmerer (2000)	
				V_{cmax}	V_{pmax}	V_{cmax}	V_{pmax}
Maize	CT	WW	25	29.9±2.3b	133.8±4.3	29.7±2.2	112.8±3.8*
Maize	CT	WD	25	22.5±0.3a	81.1±3.5	22.3±0.3	69.5±2.6*
Maize	CT	WW	38	38.7±1.0b	141.1±5.9	41.5±0.9	117.5±6.4*
Maize	CT	WD	38	33.6±1.7a	82.6±6.9	37.5±0.8	62.0±4.4*
Maize	HT	WW	25	14.4±1.8a	44.9±4.7	14.1±1.8	38.4±4.1
Maize	HT	WD	25	22.7±1.2b	52.8±8.2	22.2±1.2	46.8±7.0
Maize	HT	WW	38	31.7±0.9a	219.5±23.7	32.6±1.1	182.0±15.0
Maize	HT	WD	38	31.1±2.2a	158.7±18.6	32.2±2.2	141.4±10.0

Sensitivity and acclimation capacity to high temperature and water deficit in rice, wheat and maize

A temperature sensitivity index (TSI, Table 1) was calculated for the main photosynthetic parameters as the ratio between the value at CT-25°C and that at CT-38°C. The photosynthetic machinery of maize was particularly resistant to the sudden increase in the temperature of measurement in CT-grown plants, both under WW and WD. By contrast, A_N, g_s and g_m were affected by short-term heat stress in rice and wheat (as denoted by the asterisks), although relative sensitivity was dependent on the irrigation treatment. Irrespective of the irrigation treatment, wheat was the unique species with TSI > 1 for J_{max}, and rice presented the lowest TSI for V_{cmax} (i.e., the largest increment due to the increase in the temperature of measurement). R_{dark} was the most sensitive parameter to the increase in the temperature of measurement, particularly under WW conditions.

Table 4. Temperature sensitivity index (TSI) for the net CO_2 assimilation rate (A_N), stomatal conductance (g_s), mesophyll conductance (g_m), maximum velocity of Rubisco carboxylation (V_{cmax}), maximum rate of electron transport (J_{max}), maximum velocity of PEPC carboxylation (V_{pmax}) and mitochondrial respiration at pre-dawn (R_{dark}). TSI was calculated, under both well-watered (WW) and water deficit (WD) conditions, as the ratio between the values from plants grown and measured at 25ºC and those from plants grown at CT and measured at 38ºC (TSI = (CT-25ºC) / (CT-38ºC)). The asterisk indicates significant differences between CT-25ºC and CT-38ºC. Values are means ± standard errors (n = 5).

Parameter	WW			WD		
	Rice	Wheat	Maize	Rice	Wheat	Maize
A_N	1.6±0.2*	1.7±0.2*	0.9±0.1	0.8±0.2	2.7±0.8*	0.9±0.2
g_s	1.7±0.3*	1.0±0.2[a]	0.8±0.1	0.9±0.3	1.7±0.3*	0.9±0.2
g_m	2.5±0.9*	1.7±0.2*	-	1.2±0.4	4.8±1.3*	-
V_{cmax}	0.6±0.1*	0.9±0.1	0.8±0.1	0.4±0.1*	1.0±0.1	0.7±0.1[b]
J_{max}	0.9±0.1	1.2±0.1	-	0.7±0.1*	1.6±0.1*	-
V_{pmax}	-	-	1.0±0.1	-	-	1.0±0.1
R_{dark}	0.2±0.1*	0.2±0.1*	0.1±0.1*	0.4±0.1*	0.3±0.1*	0.3±0.1*

The temperature acclimation index (TAI, Table 5) provides a tool for comparison of plants grown and measured at the two temperatures (CT-25°C and HT-38°C). Under WW, wheat was the unique species with TAI < 1 for A_N, and both rice and wheat presented TAI > 1 for V_{cmax}, while maize TAI for V_{pmax} was also > 1 (Table 5). Under WD, maize was the unique species with TAI for A_N significantly higher than 1, and all three species presented TAI > 1 for g_s and V_{cmax}, as well as maize for V_{pmax}. TAI for R_{dark} was not significantly different from 1 under WD in any of the species, but increased in all of them under WW conditions. In wheat WW, TAI < 1 for A_N and TAI > 1 for R_{dark}, suggesting a lower capacity of acclimation to increased temperature, which may have detrimental effects on the plant carbon balance in this species.

Table 5. Temperature acclimation index (TAI) for the net CO_2 assimilation rate (A_N), stomatal conductance (g_s), mesophyll conductance (g_m), maximum velocity of Rubisco carboxylation (V_{cmax}),

maximum rate of electron transport (J_{max}), maximum velocity of PEPC carboxylation (V_{pmax}) and mitochondrial respiration at pre-dawn (R_{dark}). TAI was calculated, under both well-watered (WW) and water deficit (WD) conditions, as the ratio between the values from plants grown and measured at 38ºC and those from plants grown and measured at 25ºC (TAI = (HT-38ºC) / (CT-25ºC)). The asterisk indicates significant differences between CT-25ºC and CT-38ºC. Values are means ± standard errors (n = 5).

Parameter	WW			WD		
	Rice	Wheat	Maize	Rice	Wheat	Maize
A_N	0.9±0.1	0.7±0.1*	1.0±0.1	1.2±0.4	0.9±0.1	1.3±0.1*
g_s	1.3±0.1*	0.8±0.1	1.1±0.1	1.9±0.6[a]	2.4±0.5*	1.3±0.1*
g_m	0.6±0.1	0.8±0.1	-	0.8±0.5	0.2±0.1*	-
V_{cmax}	2.2±0.1*	1.9±0.2*	1.1±0.1	2.6±0.3[b]	1.6±0.1*	1.4±0.1*
J_{max}	1.2±0.1	1.1±0.1	-	1.2±0.2	0.9±0.1[a]	-
V_{pmax}	-	-	1.6±0.2*	-	-	2.2±0.4*
R_{dark}	2.1±1.0*	5.5±1.3*	2.3±0.3*	0.8±0.1	0.8±0.2	1.2±0.2

Discussion

The present work addresses an experimental comparison of long-term responses to increased temperature, VPD and drought stress in wheat, rice and maize, aiming to improve our understanding of how these three major global crops will respond to future climate. In addition, we take profit of the obtained data as well as of the determination of Rubisco *in vitro* kinetics for each species and condition to check the validity of commonly employed 'universal Rubisco constants' to parameterize photosynthesis models in different species. These two focusses will be discussed independently in the next sections.

Long-term acclimation to high temperature and drought stress in three important global crops

Plants grown and measured at 25ºC under well-watered conditions (CT-WW-25ºC) showed similar values for A_N in the three species (Fig. 1). However, WD resulted in a significant decrease of A_N in all three species, the strongest effect being observed in rice and the mildest in maize. In the two C_3 species, these limitations were mostly due to stomatal conductance (g_s), which largely decreased in both species, while the mesophyll

conductance to CO_2 (g_m) decreased under water deficit in rice but apparently increased in wheat. Parameters reflecting photosynthetic activity (V_{cmax}, J_{max}) were largely unaffected by WD (Fig. 2), similar to what is often found in C_3 species (Flexas *et al.* 2002, 2006b; Galmés *et al.* 2007b). In the C_4 maize, in contrast, the drought stress-induced decrease in A_N was due to combined decreases of g_s and V_{pmax}, but not V_{cmax} (Figs. 1 and 2), also in agreement with previous reports in C_4 plants (Lal & Edwards 1996; Carmo-Silva *et al.* 2010).

When WW plants grown at 25°C were measured at 38°C, significant depressions of photosynthesis were also observed in all species but maize, although these effects were of smaller magnitude than those induced by WD except in wheat, where the two stresses induced responses of similar magnitude (Fig. 1). These results may suggest that, of the three crops, rice is the most sensitive species to drought stress and wheat is the most sensitive to increased measuring temperature, while maize would be the less sensitive to both drought stress and increased measuring temperature (see also Table 4). In many studies, short-term responses are taken as evidence to predict the future photosynthetic performance of a given species under a changing climate. However, there are at least two factors that can bias these responses: (i) interactions between stresses, and (ii) acclimation in the long-term (Centritto, Lucas & Jarvis 2002; Flexas *et al.* 2006a; Vile *et al.* 2012; Cheesman & Winter 2013). Regarding interactions, these are evidenced by measuring at 38°C plants grown at 25°C and subjected to water deficit (CT-WD-38°C). In rice, A_N values were somewhat larger under WD when measured at 38°C than at 25°C, to the extent that at 38°C the effect of WD was only marginally significant (Fig. 1). In other words, in rice the high measuring temperature and drought stress interact somehow to increase photosynthesis as compared to measuring drought plants at lower temperature. In wheat, in contrast, the interaction of high measuring temperature and WD resulted in a negative potentiation of the effects of both stresses, so that photosynthesis when the two factors were applied was about half of that observed under either treatment taken separately (Fig. 1).

Considering the comparison of WW plants measured at 25°C and WD plants measured at 38°C, it can no longer be considered that rice is more drought stress sensitive and wheat more temperature sensitive. Instead, both species are similarly sensitive to the combination of high temperature and drought stress. This result illustrates how short-term studies observing the response to isolated stresses may fail to

reproduce plant responses to the most complex, combined stress conditions that are often experienced in the field (Shah & Paulsen 2003; Prasad, Staggenborg & Ristic 2008; Vile *et al.* 2012; Tozzi, Easlon & Richards 2013). In maize, the combination of high temperature and WD resulted in photosynthesis rates only marginally lower than those displayed by WW plants measured at low temperature, as expected for a C_4 species (Edwards *et al.* 2001; Crafts-brandner & Salvucci 2002; Osborne & Beerling 2006).

Long-term acclimation responses may further confound the predictive value of short-term observations. Acclimation was evident for the three species. Values of A_N were very similar between plants grown and measured at 25°C and those grown and measured at 38°C (i.e. TAI close to 1, Table 5), both under WW and WD conditions (Fig. 1a and 1b). Only in WW wheat was A_N lower in plants grown and measured at high than at low temperature, and in WD maize A_N was higher in plants grown and measured at high than at low temperature, confirming the adaptation of these two species to cool and hot temperature conditions, respectively (Hikosaka *et al.* 2006; Nagai & Makino 2009; Yamori *et al.* 2009). A similar acclimation to growth temperature – i.e., A_N is kept constant – has also been observed in poplar (Silim *et al.* 2010). Interestingly, the similar acclimation of photosynthesis to high temperature in the three species was due to different homeostatic mechanisms. For instance, in both WW and WD rice and WD wheat, the same A_N at the two temperatures was achieved by increasing g_s and V_{cmax} but decreasing g_m (Figs. 1 and 3). In WW wheat, however, a lower A_N was observed at high temperature despite increased V_{cmax}, which was in part attributable to large increases in respiration (Fig. 2). These results indicate that changing climate may result in species-dependent changes in the ratios between biochemical and diffusive parameters even in cases where net photosynthesis does not change.

In summary, the present results illustrate that the photosynthetic responses to climate conditions – e.g. drought stress and increased temperature and VPD – differ when analyzed in the short or long term, although in a manner that may be species-dependent. Therefore, it is necessary to be cautious when deriving generalizations or predictions from short-term studies with few species subjected to isolated stress conditions. Rather, detailed long-term experiments with different species and stress interactions are urged for a better understanding of crop responses to withstanding climate change conditions.

Species-specific Rubisco kinetics and their effects on accurate parameterization of C_3 and C_4 photosynthesis models

Photosynthesis models such as that of Farquhar *et al.* (1980) for C_3 plants, or that of von Caemmerer (2000) for C_4 plants, allow estimation of important biochemical traits such as the maximum velocity of Rubisco carboxylation (V_{cmax}) and the maximum rate of electron transport (J_{max}) in C_3 plants, and the maximum velocities of PEP carboxylase (V_{pmax}) and Rubisco (V_{cmax}) carboxylations in C_4 species (von Caemmerer 2013). While this parameterization was originally applied on a C_i basis (Berry & Björkman 1980; Farquhar *et al.* 1980), it is now widely recognized that correct parameterization should take into account the CO_2 concentration at the Rubisco site inside chloroplasts (C_c), for which knowledge of the mesophyll conductance to CO_2 (g_m) is required. For instance, a recent survey on 130 species reveals that assuming infinite g_m underestimates, on average, V_{cmax} by as much as 75% and J_{max} by 60% (Gu & Sun 2014; Sun *et al.* 2014). Under severe drought stress conditions the underestimations may be even larger (Flexas *et al.* 2006b). On the other hand, g_m decreases under drought stress (Flexas *et al.* 2002; Galmés *et al.* 2007a; Galle *et al.* 2009; Hu *et al.* 2010) and increases with temperature, at least within certain ranges (Silim *et al.* 2010; Evans & von Caemmerer 2013; Walker *et al.* 2013). Therefore, to correctly parameterize photosynthesis, g_m should be precisely determined for plants at each experimental condition and measurement temperature.

Several problems with existing methods for the estimation of g_m have been raised recently (Tholen *et al.* 2012; Gu & Sun 2014). On one hand, the estimated g_m may not reflect a purely diffusion conductance, because A_N is not a simple but composed CO_2 net flux combining photosynthesis, photorespiration and mitochondrial respiration, and these three processes move CO_2 along different distances and diffusion pathways (Tholen *et al.* 2012). On the other hand, apparent responses of g_m to varying light and CO_2 may be artefactual due to type I errors, consequence of analysing the dependence of g_m on variables that are explicitly included in the equations used to calculate g_m (Gu & Sun 2014). These type of errors should affect only methods that estimate different g_m values at any given C_i (i.e. Harley and Yin) but not methods that solve for a single g_m estimate along a C_i gradient (i.e. Ethier and Livingston). The fact that the estimates based on these three different methods show a good agreement (Table S1) in most cases may dismiss the importance of these errors in the present study, but

the fact that different values are obtained with different methods for some treatments suggest that potential errors cannot be completely ruled out.

Hence, while recognizing that some of the presented values may perhaps not reflect *a true* g_m, we can still use the obtained values to check for the effects of species-specific differences in Rubisco kinetic constants and their temperature response and acclimation on the parameterization of photosynthesis models. This is because in addition to precise knowledge on g_m and its temperature dependency, *a priori* knowledge of Rubisco kinetic constants ($S_{c/o}$, K_c and K_o), as well as their temperature dependencies, is required to parameterize photosynthesis models. Since these constants are unknown for Rubiscos from many species, it is becoming a common practice to use 'standard' constants for any given species. The most commonly used 'standard' Rubisco kinetics and temperature functions are those for tobacco as obtained by Bernacchi *et al.* (2002). However, it is well documented that significant differences occur among species in Rubisco kinetics (e.g., Galmés *et al.* 2005; Savir *et al.* 2010; Galmés *et al.* 2014), and a recent paper has pointed out that these differences indeed result in significant biases in model parameterization (Walker *et al.* 2013). These authors also indicate that *in vitro* Rubisco kinetics may not accurately describe the operation of Rubisco under physiological conditions, due to degradation of the enzyme during extraction or differences in the *in vitro* assay conditions compared to the chloroplast stroma. While the latter may be true, degradation of Rubisco during the extraction may affect quantitatively absolute parameters, such as the maximum Rubisco activity or Rubisco concentration, but does not affect relative values such as $S_{c/o}$, K_c and K_o. Determination of *in vivo* kinetics for a large number of species with different functional types, as urged by Walker *et al.* (2013), may not be accomplished in the short term, as such experiments require the use of mutants with low Rubisco contents for each species, growth under low CO_2 concentrations, and the use of gas exchange measurements at different oxygen partial pressures, i.e. plant material that is yet to be created and techniques that are not readily available except in a few laboratories. In contrast, measuring *in vitro* kinetic constants of Rubisco is easier and less time consuming, so that a number of different species can be characterised in a reasonable time. Therefore, we propose using Γ^* derived from *in vitro* $S_{c/o}$ measured in each species at different temperatures to first estimate g_m and, then, parameterize photosynthesis from A_N-C_c curves using the species and temperature specific *in vitro* kinetics of Rubisco rather than 'standard' values determined for model species.

This approach was used for the three species studied here and the *in vitro* values for Γ*, K_c and K_o are within the range of values obtained *in vivo* for tobacco and Arabidopsis by Walker *et al.* (2013), suggesting that using *in vitro* values is a valid approach to estimate Rubisco constants comparable to those operating *in vivo*. Rubisco from C_4 maize had a lower affinity for CO_2 (i.e., higher K_c) than the Rubisco from C_3 rice and wheat (Table 1), in agreement with previous reports (Christin *et al.* 2008; Savir *et al.* 2010; Whitney *et al.* 2011). At 25°C, differences in Γ* and K_o between species and photosynthetic mechanisms were non-significant, indicative that maize Rubisco presents a higher maximum catalytic turnover for the carboxylation reaction (k_{cat}^c). The response of Rubisco kinetics to increased temperature followed trends already described in the literature, both *in vivo* (Brooks & Farquhar 1985; Bernacchi *et al.* 2001; Walker *et al.* 2013) and *in vitro* (Badger & Collatz 1977; Jordan & Ogren 1984; Lehnherr, Mächler & Nösberger 1985; Galmés *et al.* 2005), with increases in K_c, K_o, K_c/K_o and Γ* (Table 1). The relative increase in Γ* with temperature was lower in wheat than in maize and rice. As far as we know, there are no previous reports on the temperature dependence of K_p. Previous investigations on the effect of temperature on C_4 photosynthesis parameters assumed constant K_p (Massad *et al.* 2007). In the present study, K_p decreased with increasing temperature, although to a lesser extent than K_c (Table 1). This fact, together with the higher temperature-driven increase of V_{pmax} as compared to that of V_{cmax} (Fig. 4), points suggests increased Rubisco limitations for C_4 photosynthesis at high temperatures.

Using each species Rubisco constants resulted in model parameterization estimates that in many cases differ significantly from those obtained using the 'standard' constants by Bernacchi *et al.* (2002) in C_3 plants. These differences were a generalised 10-20% overestimation of V_{cmax} and a largely variable underestimation of J_{max} (Table 2), with strongly biased V_{cmax}/J_{max} ratios. The magnitude of these discrepancies, which is similar to that found by Walker *et al.* (2013), is very significant, especially considering that all the species compared (tobacco and Arabidopsis in Walker's study, rice and wheat here) are relatively close, i.e., they are all herbaceous angiosperms. It is likely that even broader deviations would occur when using 'standard' tobacco kinetics to parameterize more distant species, like woody gymnosperms, ferns or mosses. Part of this bias in the parameterization of V_{cmax} and J_{max} is due to bias in the estimation of g_m, as indicated by the significant differences

obtained between the g_m values estimated using the Rubisco kinetic values from the present study and those reported in Bernacchi *et al.* (2001, 2002). These differences were observed regardless of the method (i.e., Harley, Ethier and Livingstone or Yin) used to estimate g_m (compare Table S1 and S2). In addition, this work shows that species and temperature specific kinetics of PEPC and Rubisco are required for accurate photosynthesis parameterization in the C_4 maize, in particular for estimating V_{pmax} (Table 3).

Table S2. Comparison of the mesophyll conductance (g_m, mol m^{-2} s^{-1}) estimated from three different methods: Harley *et al.* (1992), Ethier & Livingston (2004), Yin *et al.* (2009), in rice and wheat plants grown at CT and HT, under WW and WD conditions, and measured at 25ºC and 38ºC. The values for the Rubisco kinetic parameters required in the three methods were obtained at 25ºC and 38ºC from Bernacchi *et al.* (2001, 2002). Values are means ± standard errors (n = 5). Different letters denote statistically significant differences by Duncan analysis ($P < 0.05$) among the three methods within the same temperature and irrigation treatments.

Species	Growth T (ºC)	Irrigation Treatment	Measurement T (ºC)	g_m Harley's method	g_m Ethier's method	g_m Yin's method
Rice	CT	WW	25	0.354±0.037^{a}	0.306±0.025^{a}	0.317±0.050^{a}
Rice	CT	WD	25	0.217±0.090^{a}	0.121±0.016^{a}	0.087±0.018^{a}
Rice	CT	WW	38	0.464±0.098^{b}	0.188±0.025^{a}	0.132±0.020^{a}
Rice	CT	WD	38	0.152±0.011^{a}	0.242±0.055^{a}	0.114±0.031^{a}
Rice	HT	WW	25	0.273±0.025^{a}	0.330±0.045^{a}	0.253±0.055^{a}
Rice	HT	WD	25	0.115±0.019^{a}	0.245±0.077^{ab}	0.365±0.020^{b}
Rice	HT	WW	38	0.347±0.116^{a}	0.258±0.034^{a}	0.299±0.009^{a}
Rice	HT	WD	38	0.170±0.050^{a}	0.234±0.080^{a}	0.215±0.027^{a}
Wheat	CT	WW	25	0.203±0.010^{a}	0.196±0.017^{a}	0.170±0.005^{a}
Wheat	CT	WD	25	0.661±0.069^{b}	0.382±0.030^{a}	0.304±0.038^{a}
Wheat	CT	WW	38	0.218±0.044^{a}	0.219±0.076^{a}	0.109±0.023^{a}
Wheat	CT	WD	38	0.097±0.041^{a}	0.269±0.068^{b}	0.085±0.026^{a}
Wheat	HT	WW	25	0.137±0.007^{a}	0.181±0.019^{b}	0.136±0.009^{a}
Wheat	HT	WD	25	0.107±0.008^{a}	0.161±0.007^{b}	0.078±0.012^{a}
Wheat	HT	WW	38	0.258±0.061^{a}	0.231±0.032^{a}	0.141±0.033^{a}
Wheat	HT	WD	38	0.139±0.010^{ab}	0.189±0.038^{b}	0.073±0.010^{a}

In summary, the present results confirm and extend the conclusion by Walker *et al.* (2013) that species-specific differences in *in vivo* Rubisco parameters are large enough to significantly bias modelling of C_3 photosynthesis. It is further shown, for the first time, that differences in species-specific kinetics are large enough to bias modelling of C_4 photosynthesis. It is thus imperative to avoid using 'standard' Rubisco kinetics from tobacco to model any other species. As obtaining *in vivo* Rubisco kinetics for different species is not achievable in the short-term, we propose to use *in vitro* kinetics as determined with the methods explained here and elsewhere (Uedan & Sugiyama 1976; Bird, Cornelius & Keys 1982; Kane *et al.* 1994; Ruuska *et al.* 1998; Parry *et al.* 2007; Shay & Kubien 2013) as a proxy for *in vivo* kinetics.

Acknowledgements

This study was financed by the contract AGL2009-07999 (Plan Nacional, Spain) awarded to JG. JAP was recipient of a FPI grant from the Govern de les Illes Balears. ECS is supported by the BBSRC 20:20 Wheat Institute Strategic Program (BBSRC BB/J/00426X/1 20:20 Wheat). We thank Dr. A Diaz-Espejo (IRNAS, CSIC) for help with modelling C_4 photosynthesis, Trinidad García (radioisotope service at UIB) for technical assistance with the radioactive measurements and Dr. J. Cifre for the statistical advice.

References

Ainsworth E.A. & Ort D.R. (2010) How do we improve crop production in a warming world? *Plant Physiology* **154**, 526–30.

Atkin O.K. Scheurwater I. & Pons T. (2006) High thermal acclimation potential of both photosynthesis and respiration in two lowland Plantago species in contrast to an alpine congeneric. *Global Change Biology* **12**, 500–515.

Atkin O.K. & Macherel D. (2009) The crucial role of plant mitochondria in orchestrating drought tolerance. *Annals of Botany* **103**, 581–597.

Atkin O.K. & Tjoelker M.G. (2003) Thermal acclimation and the dynamic response of plant respiration to temperature. *Trends in Plant Science* **8**, 343–351.

Badger M. & Collatz G. (1977) Mechanism of ribulose-1,5-bisphosphate carboxylase and oxygenase reactions, with particular reference to the effect of temperature on kinetic parameters. *Carnegie Institution of Washington, Year Book* **76**, 355–361.

Bernacchi C.J. Pimentel C. & Long S.P. (2003) *In vivo* temperature response functions of parameters required to model RuBP-limited photosynthesis. *Plant, Cell and Environment* **26**, 1419–1430.

Bernacchi C.J. Portis A.R. Nakano H. von Caemmerer S. & Long S.P. (2002) Temperature Response of Mesophyll Conductance. Implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis *in vivo*. *Plant Physiology* **130**, 1992–1998.

Bernacchi C.J. Singsaas E.L. Pimentel C. Portis A.R. Jr & Long S.P. (2001) Improved temperature response functions for models of Rubisco-limited photosynthesis. *Plant, Cell and Environment* **24**, 253–259.

Berry J. & Björkman O. (1980) Photosynthetic response and adaptation to temperature in higher-plants. *Physiology and Plant Molecular Biology* **31**, 491–543.

Bird I. Cornelius M. & Keys A. (1982) Affinity of RuBP Carboxylases for Carbon Dioxide and Inhibition of the Enzymes by Oxygen. *Jounal of Experimental Botany* **33**, 1004–1013.

Brooks A. & Farquhar G.D. (1985) Effect of temperature on the CO_2/O_2 specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase and the rate of respiration in the light. *Planta* **165**, 397–406.

von Caemmerer S. (2000) *Biochemical models of leaf photosynthesis*. CSIRO, Collingwood.

von Caemmerer S. (2013) Steady-state models of photosynthesis. *Plant, Cell & Environment* **36**, 1617–1630.

Campbell C. Atkinson L. Zaragoza-Castells J. Lundmark M. Atkin O.K. & Hurry V. (2007) Acclimation of photosynthesis and respiration is asynchronous in response to changes in temperature regardless of plant functional group. *New Phytologist* **176**, 375–389.

Carmo-Silva A.E. Gore M.A. Andrade-Sanchez P. French A.N. Hunsaker D.J. & Salvucci M.E. (2012) Decreased CO_2 availability and inactivation of Rubisco limit photosynthesis in cotton plants under heat and drought stress in the field. *Environmental and Experimental Botany* **83**, 1–11.

Carmo-Silva A.E. Keys A.J. Andralojc P.J. Powers S.J. Arrabaça M.C. & Parry M.A.J. (2010) Rubisco activities, properties, and regulation in three different C_4 grasses under drought. *Journal of Experimental Botany* **61**, 2355–2366.

Carmo-Silva A.E. Powers S.J. Keys A.J. Arrabaça M.C. & Parry M.A.J. (2008) Photorespiration in C_4 grasses remains slow under drought conditions. *Plant, Cell & Environment* **31**, 925–940.

Centritto M. Lucas M.E. & Jarvis P.G. (2002) Gas exchange, biomass, whole-plant water-use efficiency and water uptake of peach (*Prunus persica*) seedlings in response to elevated carbon dioxide concentration and water availability. *Tree Physiology* **22**, 699–706.

Cheesman A.W. & Winter K. (2013) Growth response and acclimation of CO_2 exchange characteristics to elevated temperatures in tropical tree seedlings. *Journal of Experimental Botany* **64**, 3817–3828.

Christin P.A. Salamin N. Muasya A.M. Roalson E.H. Russier F. & Besnard G. (2008) Evolutionary switch and genetic convergence on *rbc*L following the evolution of C_4 photosynthesis. *Molecular Biology and Evolution* **25**, 2361–2368.

Crafts-brandner S.J. & Salvucci M.E. (2002) Sensitivity of Photosynthesis in a C_4 plant, maize, to heat stress. *Plant Physiology* **129**, 1773–1780.

Diaz-Espejo A. (2013) New challenges in modelling photosynthesis: temperature dependencies of Rubisco kinetics. *Plant, Cell & Environment* **36**, 2104–2107.

Dillaway D.N. & Kruger E.L. (2011) Leaf respiratory acclimation to climate: comparisons among boreal and temperate tree species along a latitudinal transect. *Tree Physiology* **31**, 1114–1127.

Edwards G. Furbank R.T. Hatch M. & Osmond C. (2001) What does it take to be C_4? Lessons from the evolution of C_4 photosynthesis. *Plant Physiology* **125**, 46–49.

Ethier G.J. & Livingston N.J. (2004) On the need to incorporate sensitivity to CO_2 transfer conductance into the Farquhar-von Caemmerer-Berry leaf photosynthesis model. *Plant, Cell and Environment* **27**, 137–153.

Evans J.R. & von Caemmerer S. (2013) Temperature response of carbon isotope discrimination and mesophyll conductance in tobacco. *Plant, Cell & Environment* **36**, 745–756.

Farquhar G.D., von Caemmerer S. & Berry J.A. (1980) A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. *Planta* **149**, 78–90.

Flexas J. Barón M., Bota J. Ducruet J.M. Gallé A. Galmés J. … Medrano H. (2009) Photosynthesis limitations during water stress acclimation and recovery in the drought-adapted Vitis hybrid Richter-110 (*V. berlandieri*x*V. rupestris*). *Journal of Experimental Botany* **60**, 2361–2377.

Flexas J. Bota J. Escalona J. Sampol B. & Medrano H. (2002) Effects of drought on photosynthesis in grapevines under field conditions: an evaluation of stomatal and mesophyll limitations. *Functional Plant Biology* **29**, 461–471.

Flexas J. Bota J. Galmés J. Medrano H. & Ribas-Carbó M. (2006a) Keeping a positive carbon balance under adverse conditions: responses of photosynthesis and respiration to water stress. *Physiologia Plantarum* **127**, 343–352.

Flexas J. Díaz-Espejo A. Berry J.A Cifre J. Galmés J. Kaldenhoff R. Medrano H. & Ribas-Carbó M. (2007) Analysis of leakage in IRGA's leaf chambers of open gas exchange systems: quantification and its effects in photosynthesis parameterization. *Journal of Experimental Botany* **58**, 1533–1543.

Flexas J. Niinemets Ü. Gallé A. Barbour M.M. Centritto M. Diaz-Espejo A. … Medrano H. (2013) Diffusional conductances to CO_2 as a target for increasing photosynthesis and photosynthetic water-use efficiency. *Photosynthesis Research* **117**, 45–59.

Flexas J. Ribas-Carbó M. Bota J. Galmés J. Henkle M. Martínez-Cañellas S. Medrano H. (2006b) Decreased Rubisco activity during water stress is not induced by decreased relative water content but related to conditions of low stomatal conductance and chloroplast CO_2 concentration. *New Phytologist* **172**, 73–82.

Galle A. Florez-Sarasa I. Tomas M. Pou A. Medrano H. Ribas-Carbó M. & Flexas J. (2009) The role of mesophyll conductance during water stress and recovery in tobacco (*Nicotiana sylvestris*): acclimation or limitation? *Journal of Experimental Botany* **60**, 2379–2390.

Galmés J. Flexas J., Keys A. Cifre J. Mitchell R. Madgwick P. … Parry M.A.J. (2005) Rubisco specificity factor tends to be larger in plant species from drier habitats and in species with persistent leaves. *Plant, Cell & Environment* **28**, 571–579.

Galmés J. Flexas J. Savé R. & Medrano H. (2007a) Water relations and stomatal characteristics of Mediterranean plants with different growth forms and leaf habits: responses to water stress and recovery. *Plant and Soil* **290**, 139–155.

Galmés J. Kapralov M.V. Andralojc P.J. Conesa M.À. Keys A.J. Parry M.A.J. & Flexas J. (2014) Expanding knowledge of the Rubisco kinetics variability in plant species: environmental and evolutionary trends. *Plant, Cell & Environment*.

Galmés J. Medrano H. & Flexas J. (2006) Acclimation of Rubisco specificity factor to drought in tobacco: discrepancies between *in vitro* and *in vivo* estimations. *Journal of Experimental Botany* **57**, 3659–67.

Galmés J. Medrano H. & Flexas J. (2007b) Photosynthetic limitations in response to water stress and recovery in Mediterranean plants with different growth forms. *The New Phytologist* **175**, 81–93.

Galmés J. Ribas-Carbó M. Medrano H. & Flexas J. (2007c) Response of leaf respiration to water stress in Mediterranean species with different growth forms. *Journal of Arid Environments* **68**, 206–222.

Galmés J. Ribas-Carbó M. Medrano H. & Flexas J. (2011) Rubisco activity in Mediterranean species is regulated by the chloroplastic CO_2 concentration under water stress. *Journal of Experimental Botany* **62**, 653–665.

Genty B. Briantais J. & Baker N. (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* **990**, 87–92.

Gornall J. Betts R. Burke E. Clark R. Camp J. Willett K. & Wiltshire A. (2010) Implications of climate change for agricultural productivity in the early twenty-first century. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **365**, 2973–2989.

Grassini P. Eskridge K.M. & Cassman K.G. (2013) Distinguishing between yield advances and yield plateaus in historical crop production trends. *Nature Communications* **4**, 2918.

Gu L. & Sun Y. (2014) Artefactual responses of mesophyll conductance to CO_2 and irradiance estimated with the variable J and online isotope discrimination methods. *Plant, Cell & Environment* **37**, 1231–1249.

Harley P.C. Loreto F. Di Marco G. & Sharkey T.D. (1992) Theoretical Considerations when estimating the Mesophyll conductance to CO_2 flux by analysis of the response of photosynthesis to CO_2. *Plant Physiology* **98**, 1429–1436.

Hikosaka K. Ishikawa K. Borjigidai A. Muller O. & Onoda Y. (2006) Temperature acclimation of photosynthesis: mechanisms involved in the changes in temperature dependence of photosynthetic rate. *Journal of Experimental Botany* **57**, 291–302.

Hu L. Wang Z. & Huang B. (2010) Diffusion limitations and metabolic factors associated with inhibition and recovery of photosynthesis from drought stress in a C_3 perennial grass species. *Physiologia Plantarum* **139**, 93–106.

IPCC (2013) *Working Group I Contribution to the IPCC Fifth Assessment Report Climate Change 2013: The Physical Science Basis Summary for Policymakers*.

Jordan D.B. & Ogren W.L. (1984) The CO_2/O_2 specificity of ribulose 1,5-bisphosphate carboxylase/oxygenase. *Planta* **161**, 308–313.

Kane H. Viil J. Entsch B. Paul K. Matthew K. & Andrews T. (1994) An improved method for measuring the CO_2/O_2 specificity of ribulosebisphosphate carboxylase-oxygenase. *Australian Journal of Plant Physiology* **21**, 449–461.

Kattge J. & Knorr W. (2007) Temperature acclimation in a biochemical model of photosynthesis: a reanalysis of data from 36 species. *Plant, Cell & Environment* **30**, 1176–1190.

Krall J. & Edwards G. (1992) Relationship between photosystem II activity and CO_2 fixation in leaves. *Physiologia Plantarum* **86**, 180–187.

Laisk A. & Loreto F. (1996) Determining photosynthetic parameters from leaf CO_2 exchange and chlorophyll fluorescence. *Plant Physiology* **110**, 903–912.

Lal A. & Edwards G. (1996) Analysis of inhibition of photosynthesis under water stress in the C_4 species *Amaranthus cruentus* and *Zea mays*: electron transport, CO_2 fixation and carboxylation. *Functional Plant Biology* **23**, 403–412.

Lehnherr B. Mächler F. & Nösberger J. (1985) Influence of Temperature on the ratio of ribulose bisphosphate carboxylase to oxygenase activities and on the ratio of photosynthesis to photorespiration of leaves. *Journal of Experimental Botany* **36**, 1117–1125.

Long S. Ainsworth E.A. Leakey A. Nösberger J. & Ort D. (2006) Food for thought: lower-than-expected crop yield stimulation with rising CO_2 concentrations. *Science* **312**, 1918–1921.

Loriaux S.D. Avenson T.J. Welles J.M. McDermitt D.K. Eckles R.D. Riensche B. & Genty B. (2013) Closing in on maximum yield of chlorophyll fluorescence using a single multiphase flash of sub-saturating intensity. *Plant, Cell & Environment* **36**, 1755–1770.

Makino A. (2011) Photosynthesis, grain yield, and nitrogen utilization in rice and wheat. *Plant Physiology* **155**, 125–129.

Martins S.C.V. Galmés J. Molins A. & Da Matta F.M. (2013) Improving the estimation of mesophyll conductance to CO_2: on the role of electron transport rate correction and respiration. *Journal of Experimental Botany* **64**, 3285–3298.

Massad R.S. Tuzet A. & Bethenod O. (2007) The effect of temperature on C_4-type leaf photosynthesis parameters. *Plant, Cell & Environment* **30**, 1191–1204.

Mittler R. (2006) Abiotic stress, the field environment and stress combination. *Trends in Plant Science* **11**, 15–19.

Nagai T. & Makino A. (2009) Differences between rice and wheat in temperature responses of photosynthesis and plant growth. *Plant & Cell Physiology* **50**, 744–755.

Osborne C.P. & Beerling D.J. (2006) Nature's green revolution: the remarkable evolutionary rise of C_4 plants. *Philosophical transactions of the Royal Society of London. Series B, Biological Sciences* **361**, 173–194.

Parry M.A.J. Madgwick P.J. Carvalho J.F.C. & Andralojc P.J. (2007) Prospects for increasing photosynthesis by overcoming the limitations of Rubisco. *The Journal of Agricultural Science* **145**, 31–43.

Parry M.A.J. Keys A.J. & Gutteridge S. (1989) Variation in the specificity factor of C_3 higher plant Rubisco determined by the total consumption of ribulose-P_2. *Journal of Experimental Botany* **40**, 317–320.

Pittermann J. & Sage R.F. (2001) The response of the high altitude C_4 grass *Muhlenbergia montana* (Nutt.) A.S. Hitchc. to long- and short-term chilling. *Journal of Experimental Botany* **52**, 829–838.

Prasad P. Staggenborg S. & Ristic Z. (2008) Impacts of drought and/or heat stress on physiological, developmental, growth, and yield processes of crop plants. In *Modeling Water Stress Effects on Plant Growth Processes*. (Eds L. Ahuja & S. Saseendran), pp. 301–356. Madison, Wisconsin.

Rodríguez-Calcerrada J. Atkin O.K. Robson T.M. Zaragoza-Castells J. Gil L. & Aranda I. (2010) Thermal acclimation of leaf dark respiration of beech seedlings experiencing summer drought in high and low light environments. *Tree Physiology* **30**, 214–224.

Ruuska S. Andrews T. Badger M. Hudson G. Laisk A. Price G. & von Caemmerer (1998) The interplay between limiting processes in C_3 photosynthesis studied by rapid-response gas exchange using transgenic tobacco impaired in photosynthesis. *Australian Journal of Plant Physiology* **25**, 859–870.

Sage R.F. & Kubien D.S. (2007) The temperature response of C_3 and C_4 photosynthesis. *Plant, Cell & Environment* **30**, 1086–1106.

Salvucci M.E. & Crafts-brandner S.J. (2004) Relationship between the heat tolerance of photosynthesis and the thermal stability of Rubisco Activase in plants from contrasting thermal environments. *Plant Physiology* **134**, 1460–1470.

Savir Y. Noor E. Milo R. & Tlusty T. (2010) Cross-species analysis traces adaptation of Rubisco toward optimality in a low-dimensional landscape. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 3475–3480.

Scafaro A.P. von Caemmerer S. Evans J.R. & Atwell B.J. (2011) Temperature response of mesophyll conductance in cultivated and wild Oryza species with contrasting mesophyll cell wall thickness. *Plant, Cell & Environment* **34**, 1999–2008.

Schultz H. (1996) Leaf absorptance of visible radiation in *Vitis vinifera* L.: estimates of age and shade effects with a simple field method. *Scientia Horticulturae* **66**, 93–102.

Shah N.H. & Paulsen G.M. (2003) Interaction of drought and high temperature on photosynthesis and grain-filling of wheat. *Plant and Soil* **257**, 219–226.

Shay P.E. & Kubien D.S. (2013) Field analysis of photoprotection in co-occurring cool climate C_3 and C_4 grasses. *Physiologia Plantarum* **147**, 316–328.

Silim S.N. Ryan N. & Kubien D.S. (2010) Temperature responses of photosynthesis and respiration in *Populus balsamifera* L.: acclimation versus adaptation. *Photosynthesis Research* **104**, 19–30.

Sun Y. Gu L. Dickinson R.E. Pallardy S.G. Baker J. Cao Y. … Winter K. (2014) Asymmetrical effects of mesophyll conductance on fundamental photosynthetic parameters and their relationships estimated from leaf gas exchange measurements. *Plant, Cell & Environment* **37**, 978–994.

Tholen D. Ethier G. Genty B. Pepin S. & Zhu X.G. (2012) Variable mesophyll conductance revisited: theoretical background and experimental implications. *Plant, Cell & Environment* **35**, 2087–2103.

Tosens T. Niinemets Ü. Vislap V. Eichelmann H. & Castro Díez P. (2012) Developmental changes in mesophyll diffusion conductance and photosynthetic capacity under different light and water availabilities in *Populus tremula*: how structure constrains function. *Plant, Cell & Environment* **35**, 839–856.

Tozzi E.S. Easlon H.M. & Richards J.H. (2013) Interactive effects of water, light and heat stress on photosynthesis in Fremont cottonwood. *Plant, Cell & Environment* **36**, 1423–1434.

Uedan K. & Sugiyama T. (1976) Purification and characterization of phosphoenolpyruvate carboxylase from maize leaves. *Plant Physiology* **57**, 906–910.

Vile D. Pervent M. Belluau M. Vasseur F. Bresson J. Muller B. Granier C. & Simonneau T. (2012) Arabidopsis growth under prolonged high temperature and

water deficit: independent or interactive effects? *Plant, Cell & Environment* **35**, 702–718.

Walker B. Ariza L.S. Kaines S. Badger M.R. & Cousins A.B. (2013) Temperature response of *in vivo* Rubisco kinetics and mesophyll conductance in *Arabidopsis thaliana*: comparisons to *Nicotiana tabacum*. *Plant, Cell & Environment* **36**, 2108–2119.

Walters R.G. (2005) Towards an understanding of photosynthetic acclimation. *Journal of Experimental Botany* **56**, 435–447.

Whitney S.M. Sharwood R.E. Orr D. White S.J. Alonso H. & Galmés J. (2011) Isoleucine 309 acts as a C_4 catalytic switch that increases ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylation rate in Flaveria. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 14688–14693.

Yamori W. Noguchi K. Hikosaka K. & Terashima I. (2009) Cold-tolerant crop species have greater temperature homeostasis of leaf respiration and photosynthesis than cold-sensitive species. *Plant & Cell Physiology* **50**, 203–215.

Yamori W. Noguchi K. & Terashima I. (2005) Temperature acclimation of photosynthesis in spinach leaves: analyses of photosynthetic components and temperature dependencies of photosynthetic partial reactions. *Plant, Cell and Environment* **28**, 536–547.

Yamori W. Takahashi S. Makino A. Price G.D. Badger M.R. & von Caemmerer S. (2011) The roles of ATP synthase and the cytochrome b_6/f complexes in limiting chloroplast electron transport and determining photosynthetic capacity. *Plant Physiology* **155**, 956–962.

Yin X. Struik P.C. Romero P. Harbinson J. Evers J.B. Van Der Putten P.E.L. & Vos J. (2009) Using combined measurements of gas exchange and chlorophyll fluorescence to estimate parameters of a biochemical C_3 photosynthesis model: a critical appraisal and a new integrated approach applied to leaves in a wheat (*Triticum aestivum*) canopy. *Plant, Cell & Environment* **32**, 448–464.

Chapter 5

RUBISCO AND RUBISCO ACTIVASE ROLE IN THE BIOCHEMICAL LIMITATIONS OF PHOTOSYNTHESIS

5.1. RUBISCO AND RUBISCO ACTIVASE PLAY AN IMPORTANT ROLE IN THE BIOCHEMICAL LIMITATIONS OF PHOTOSYNTHESIS IN RICE, WHEAT AND MAIZE UNDER HIGH TEMPERATURE AND WATER DEFICIT

Juan Alejandro Perdomo[1]*, Elizabete Carmo-Silva[2], Michael E. Salvucci[3], Jeroni Galmés[1]

[1]*Research Group on Plant Biology under Mediterranean Conditions, Universitat de les Illes Balears, Balearic Islands, Spain.*

[2]*Plant Biology and Crop Science, Rothamsted Research, Harpenden, AL5 2JQ, UK.*

[3]*USDA-ARS, Arid-Land Agricultural Research Center, Maricopa, AZ 85138*

***Corresponding author:** Juan Alejandro Perdomo

e-mail: alejandro.perdomo@uib.es

Fax: +34 / 971 / 173184

Phone: +34 / 971 / 259556

Abstract

To understand the effect of heat and drought on three major cereal crops, we examined the physiological and biochemical photosynthetic responses of rice, wheat and maize plants grown under long-term water deficit (WD), high temperature (HT) and the combination of both (HT-WD). Our results showed that diffusional limitations to photosynthesis prevail under WD for the C_3 species, rice and wheat. Conversely, biochemical limitations prevailed under WD for the C_4 species, maize, and under HT for all three species, being it also predominant under HT-WD in rice and maize. These biochemical limitations to photosynthesis were associated with Rubisco activity that was highly impaired at HT and under HT-WD in the three species. Decreases in Rubisco activation were not related with the amount of Rubisco and Rubisco activase (Rca), but might be attributed to an inhibition of the Rca activity, as suggested by the positive correlation between Rubisco actitation state and the rate of electron transport. Likewise, the decrease in Rubisco activation at HT was greatly correlated with impairments in the net CO_2 assimilation rate (A_N). Overall, the results obtained in this study highlight the importance to consider the responses of Rubisco to HT and WD to improve the photosynthetic performance of crops against the detrimental effect of climate change in agriculture.

Introduction

As a consequence of climate change, global temperatures have increased over the last few decades and this warming trend is predicted to accelerate over the next future (IPCC 2013). Increases in global temperatures are often accompanied by alterations in precipitation patterns, with effects on the amount, intensity, frequency and type of precipitation (Dore 2005).

Heat and drought are the principal abiotic stresses limiting plant growth and crop productivity. Photosynthesis, the main physiological process driving plant growth, is highly sensitive to drought and heat stress (Chaves, Flexas & Pinheiro 2009; Mathur, Agrawal & Jajoo 2014), especially when both stresses are imposed together (Carmo-Silva *et al.* 2012; Vile *et al.* 2012; Perdomo *et al.* 2014a). Photosynthetic CO_2 assimilation can be constrained by diffusive and biochemical limitations (Flexas & Medrano 2002; Pinheiro & Chaves 2011). The diffusive limitations come as a consequence of stomatal closure (i.e., decreased stomatal conductance, g_s) and increased

leaf resistance to CO_2 transport from the atmosphere to the site of carboxylation (i.e., decreased mesophyll conductance, g_m), generally observed under mild to moderate water deficit (Chaves, Maroco & Pereira 2003; Flexas *et al.* 2004; Chaves *et al.* 2009).

The biochemical components that limit photosynthesis under water deficit are less well described than the diffusion limitations (Galmés, Medrano & Flexas 2007b). Metabolic limitations to photosynthesis under drought have been associated with impaired ATP synthesis (Tezara *et al.* 1999; Flexas *et al.* 2004), which is due to a decrease in the electron transport rate (J) (Flexas, Escalona & Medrano 1999; Galmés *et al.* 2007a). Lower ATP availability, in turn, affects ribulose-1,5-bisphosphate (RuBP) regeneration, thus limiting the rate of CO_2 fixation. The effects of drought stress on Rubisco vary depending on the species and intensity of stress; some studies reported a dramatic reduction in Rubisco activity (Parry *et al.* 2002; Zhou, Lam & Zhang 2007) while others showed little or no inhibition of the enzyme (Panković *et al.* 1999; Pelloux *et al.* 2001). A meta-analyses suggested that Rubisco did not limit photosynthesis until severe drought stress or long-term water stress was encountered (Flexas *et al.* 2006a). A recent study by Galmés *et al.* (2011) in Mediterranean species suggests that low chloroplastic CO_2 concentration (C_c) occurring under water deficit could induce de-activation of Rubisco sites.

High temperatures of measurement have been shown to affect both electron transport capacity (J_{max}) and the maximum rate of carboxylation of Rubisco (V_{cmax}) (Dreyer *et al.* 2001; Yamori *et al.* 2006, 2008). On the contrary, data in literature suggest that high temperatures of measurement do not sufficiently impair g_s and g_m to cause diffusion components as the prevailing limitation to photosynthesis (Bernacchi *et al.* 2002; Evans & von Caemmerer 2013; Walker *et al.* 2013; von Caemmerer & Evans 2014). It is well-described that moderately high temperatures impair the Rubisco activation state, which becomes the primary cause of the decrease in the photosynthetic rate (Crafts-Brandner & Salvucci 2000; Salvucci & Crafts-brandner 2004; Kim & Portis 2005; Galmés *et al.* 2013). As the temperature increases further above the thermal optimum and reaches non-physiological conditions, photosynthesis may be increasingly limited due to impairment of the biochemical integrity of the photosynthetic apparatus (Salvucci & Crafts-brandner 2004). High temperature can inhibit electron transport activity, ATP synthesis, RuBP regeneration, and the re-activation of Rubisco by its catalytic chaperone, Rubisco activase (Rca) (Schrader *et al.* 2004; Makino & Sage 2007; Yamori *et al.* 2008).

The above described effects of high temperature on the photosynthetic processes are mainly related to the temperature of measurement in plants grown at a given temperature. Although there is abundant evidence that photosynthesis can acclimate to temperature (Gunderson, Norby & Wullschleger 2000; Way & Yamori 2014; Yamori, Hikosaka & Way 2014), little is known about the effects of the growth temperature on the contributions of diffusive and biochemical limitations to photosynthesis. If biochemical limitations prevailing at high temperatures of measurement also predominate at high temperatures of growth, the analysis of Rubisco and Rca performance and thermal acclimation may provide valuable information towards the improvement of crop photosynthesis under high temperature stress.

The activity of Rubisco is regulated by Rca, which facilitates the dissociation of inhibitory sugar phosphates from the active site of Rubisco in an ATP-dependent reaction (Spreitzer & Salvucci 2002). Most species studied contain two isoforms of Rca, a shorter redox-insensitive β-isoform of 41-43 kDa and a longer redox-sensitive α-isoform of 46-48 kDa (Zhang & Portis 1999). Changes in the redox status and ADP/ATP ratio of the chloroplast modulate the activity of Rca, thereby mediating the regulation of Rubisco activation and the net CO_2 assimilation in response to the prevailing irradiance (Salvucci, Portis & Ogren 1985; Mott & Woodrow 2000; Carmo-Silva & Salvucci 2013; Scales, Parry & Salvucci 2014). The activity of Rca is extremely thermally sensitive. This enzyme becomes inactive, decreasing the rate of net CO_2 assimilation at moderately high temperatures. The decreased rate of CO_2 assimilation impairs plant growth under periods of moderate heat stress and is detrimental to crop productivity (Lobell & Asner 2003).

In the present study, we focused on analyzing the effects of long-term growth under water deficit (WD), high temperature (HT) and the combination of both (HT-WD) on the Rubisco activity and amount, Rubisco activase content and Rubisco activation state and to relate them with the relative contributions of biochemical and diffusive limitations to photosynthesis in rice, wheat and maize.

Material and Methods

Plant material, growth conditions and treatments

Rice (*Oryza sativa* L. cv. Bomba), wheat (*Triticum aestivum* L. cv. Cajeme) and maize (*Zea mays* cv. Carella) were grown from seeds in a greenhouse in 3.5 L pots containing a 70:30 mixture of horticultural substrate and perlite (granulometry A13). After two weeks, the seedlings were selected to uniform size with one plant per pot in maize, and ten plants per pot in wheat and rice. Thereafter, the plants were moved to a walk-in-growth chamber (phytotron), under controlled conditions of light intensity, photoperiod, relative humidity and temperature. Light was provided by metal halide lamps (OSRAM, Germany) placed at specific distances from the plants to obtain a photosynthetically active photon flux density (PPFD) of 500 μmol m^{-2} s^{-1}, with a photoperiod of 12 h day/12 h night. The ambient temperature and the relative humidity were monitored with portable sensors Testo 175-H1 data logger (Gerilab, Spain). The relative humidity was maintained between 40-60% using humidifiers.

For logistical reasons, the plants were grown in two sets, which were subjected to each of the two temperature treatments. A first set of plants of the three species was grown at the control temperature (CT, 25/20°C; VPD, 1.8/1.0 kPa day/night); and a second set of plants was grown at high temperature (HT, 38/33°C; VPD, 3.5/2.3 kPa day/night). For each of the temperature regimes, ten pots per species were grown at field capacity until they presented fully expanded leaves (typically two weeks). Thereafter, 20 days after germination, pots of all species were randomly assigned to two different irrigation treatments: five pots per species were maintained at 100% field capacity during the whole experiment (well-watered treatment, WW) and the other five pots were maintained at 45% field capacity (moderate water deficit treatment, WD), as determined by pot weighting every other day and compensating the daily water losses with 50% Hoagland's solution. Therefore, a total of four treatments were established: 25ºC of growth temperature and well-watered (control), 25ºC of growth temperature and water-deficit (WD), 38ºC of growth temperature and well-watered (HT) and 38ºC of growth temperature and water-deficit (HT-WD). New leaves were allowed to develop and expand under all treatments. All measurements and samples were taken at least 40 days after the water treatment was initiated (i.e., 60 days after germination) on new leaves developed completely under the treatments.

Leaf samples for biochemical measurements were collected at midday. Leaf discs of 0.5 cm^2 were quickly frozen into liquid nitrogen and stored at -80°C until extraction. These samples were used for the following determinations: Rubisco initial and total activity, activation state and amount, and Rubisco activase amount.

Gas exchange and chlorophyll* a *fluorescence measurements

All leaf gas exchange and chlorophyll *a* fluorescence measurements were performed on the youngest fully expanded leaf of each plant, using a portable gas exchange system (Li-6400-40; Li-Cor Inc., USA). The net CO_2 assimilation rate (A_N) and the stomatal conductance (g_s) were measured at mid-morning at a leaf temperature of 25ºC, saturating PPFD of 1500 µmol m^{-2} s^{-1} (provided by the light source of the Li-6400-40, with 10% blue light), a CO_2 concentration in the leaf chamber (C_a) of 400 µmol CO_2 mol^{-1} air and a relative humidity between 40 and 50%. The leaf dark respiration rate (R_{dark}) was determined at pre-dawn (i.e., shortly before the start of the light period) at a C_a of 400 µmol CO_2 mol^{-1} air. The gross CO_2 assimilation rate (A_G) was calculated from the sum between A_N and half of the R_{dark} (Bermúdez *et al*. 2012).

The fluorometer was set to multiphase pulse with factory setting, target intensity=10 and ramp depth=40% (Loriaux *et al.* 2013). The photochemical efficiency of photosystem II (Φ_{PSII}) was determined according to Genty, Briantais & Baker (1989):

$$\Phi_{PSII} = (F_m' - F_s) / F_m' \qquad [1]$$

where F_s is the steady-state fluorescence yield and F_m' the maximum fluorescence yield obtained with a light-saturating pulse of 8000 µmol m^{-2} s^{-1}.

The linear rate of electron transport (J) was calculated according to Krall & Edwards (1992):

$$J = \Phi_{PSII} \cdot PPFD \cdot \alpha \cdot \beta \qquad [2]$$

where α is the leaf absorbance and β is the partitioning of absorbed quanta between photosystems I and II. β was assumed to be 0.5 (Laisk & Loreto 1996; Tosens *et al.* 2012). α was measured for all species grown under each treatment inside a dark chamber using the light source from the Li-6400 and a spectroradiometer (HR2000CG-UV-NIR; Ocean Optics Inc., USA) for the range 325-1075 nm, as described by Schultz (1996). α values ranged between 0.87 for measurements at 25°C and 0.86 for measurements at 38°C, with non-significant differences between species and species × treatment combinations.

Estimation of C_c, C_s and g_m

From combined gas-exchange and chlorophyll *a* fluorescence measurements, the mesophyll conductance to CO_2 (g_m) was estimated for wheat and rice using the so-called variable J method (Harley *et al.* 1992). Maize has a C_4-based carbon concentrating mechanism, with inherent complexity that complicates mathematical modeling (Collatz et al., 1992; von Caemmerer & Furbank, 1999; von Caemmerer, 2000; Yin et al., 2011; Ubierna et al., 2012). Therefore, for maize g_m was considered constant at 2000 mmol m^{-2} s^{-1} (von Caemmerer 2000). The value of g_m determined for wheat and rice, both C_3 species, was used to calculate C_c by applying the equation:

$$C_c = C_i - (A_N / g_m) \quad [3]$$

The CO_2 concentration in the bundle sheath (C_s) of maize leaves was estimated from the hyperbolic function describing the A_N-C_i curves using the C_4 photosynthesis model described by von Caemmerer (2000) as detailed by Massad *et al.* (2007) and with the modifications of Carmo-Silva *et al.* (2008).

Quantification of photosynthetic limitations

To compare the relative limitations to CO_2 assimilation induced by WD, HT and the combination of both stresses, the photosynthetic limitations were partitioned into their functional components following the approach proposed by Grassi and Magnani (2005). This approach uses values for A_G, g_s and g_m (Table S1) and the maximum rate of Rubisco carboxylation (V_{cmax}). In the present study, V_{cmax} was calculated as the product of the Rubisco amount, the activation state and the carboxylase catalytic turnover rate (k_{cat}^c) measured *in vitro* at 25ºC (2.1, 2.2 and 4.1 s^{-1} for rice, wheat and maize, respectively; Perdomo *et al.* 2014b). Thereafter, the photosynthetic limitations can then be partitioned into components related to diffusion, i.e., stomatal (S_L) and mesophyll limitations (MC_L), and leaf biochemistry (B_L). The analysis of biochemical limitations in maize was restricted to the C_3 cycle activity. Data obtained under control conditions (CT-WW) was used as the reference.

Rubisco activity and amount in leaf crude extracts

Rubisco was extracted by grinding three leaf disc samples (in total 1.5 cm^2) in a mortar with 500 µL of ice-cold extraction buffer containing 50 mM Bicine-NaOH pH 8.0, 1 mM ethylene diamine tetracetic acid (EDTA), 5% polivinil pirrolidone (PVP), 6% polyethylene glycol (PEG_{4000}), 50 mM β-mercaptoethanol, 10 mM dithiothreitol (DTT) and 1% protease-inhibitor cocktail (Sigma-Aldrich Co. LLC., USA). Leaf

extracts were then centrifuged at 14000×*g* for 1 min at 4°C. The supernatant was kept at 4°C and used immediately for the measurement of Rubisco activity and amount.

The activities of Rubisco were determined by the incorporation of $^{14}CO_2$ into acid-stable products at a reaction temperature of 25°C for plants grown both at CT and HT, following the protocol described in Parry *et al.* (1997). The reaction mixture (0.5 mL) contained 100 mM Bicine-NaOH pH 8.2, 20 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (15.54 kBq μmol^{-1}) and 0.1 mM RuBP. The initial activity was determined by adding 10 µL of crude extract to the reaction mixture. The total activity was measured after incubating 10 µL of the same extract for 3 minutes with all the components except RuBP, to allow carbamylation of all available Rubisco catalytic sites, and then starting the reaction by adding RuBP. All reactions were quenched after 60 s by adding 100 µL of 10 M HCOOH. The activation state of Rubisco was obtained as the ratio between the initial and total activities. All quenched reaction mixtures were completely dried at 100ºC, the residues dissolved in 400 µL H_2O, mixed with 3.6 mL of Ultima Gold scintillation cocktail (PerkinElmer Inc., USA) and the ^{14}C products determined in a scintillation counter (LS-6500, Beckman Coulter Inc., USA).

The amount of Rubisco was measured using the electrophoresis method (Aranjuelo *et al.* 2005). One aliquot of the leaf crude extract was mixed with loading buffer, consisting of 65 mM Tris-HCl pH 6.8, 3 M sucrose, 0.6 M β-mercaptoethanol, 5% (w/v) sodium dodecylsulphate (SDS), and 0.01% bromophenol blue. Samples were heated at 96°C for 5 min and then allowed to cool at room temperature. The total soluble protein (TSP) concentration in the crude extracts was determined by the method of Bradford (1976). A volume representing 15 µg of TSP per sample (crude extract mixed with loading buffer) was loaded onto a 12.5% SDS-polyacrylamide gel (Bio-Rad Laboratories Inc., USA). This amount of protein was within the range of linear response of optical density for known concentrations of Rubisco purified from wheat (standard used for calibration). The solubilized proteins were separated by SDS–PAGE (Laemmli, 1970) using a 0.75 mm thick gel (12.5% resolving, 4% stacking). Electrophoresis was carried out at room temperature at a constant voltage (200 V). The gels were fixed in 500:150:75 (v/v/v) water–methanol–acetic acid mixture for 1 h, stained in EZ Blue Gel Staining (Sigma-Aldrich Co. LLC., USA) solution for 1 h and subsequently rinsed in water to remove excess stain. Finally, the gels were scanned with a high-resolution scanner (HP Scanjet G3010, Hewlett Packard, Spain) and the amount of large Rubisco

subunit was determined by densitometry with the image analysis software TotalLab v2005 (Nonlinear Dynamics, Durham, USA).

Rubisco activase protein amount

The relative amount of Rubisco activase (Rca) was measured by SDS–PAGE and immunoblotting (Salvucci *et al.* 2001). Soluble proteins were extracted from samples consisting of three leaf discs (1.5 cm^2) by grinding in a mortar with 500 µL of ice-cold extraction buffer containing 50 mM Tricine-NaOH pH 8.0, 10 mM EDTA, 1% PVP, 20 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µM leupeptin and 1% protease-inhibitor cocktail. The leaf extracts were centrifuged at 14000×*g* for 1 min at 4°C and 25 µL of the supernatant was rapidly added to 20 µL loading buffer (described above). After determination of the TSP concentration in the crude extracts, sample aliquots of extracts plus loading buffer corresponding to 6 µg of TSP were loaded onto a 12.5% SDS-polyacrylamide gel (Bio-Rad, Spain) and separated by electrophoresis. Serial dilutions of extracts prepared from leaf discs taken from plants of each species under control conditions were used as standards, by loading 5, 10 and 15 µg of TSP. SDS-PAGE gels were blotted onto nitrocellulose membranes in 50 mM Trizma base/50 mM boric acid for 1 h at 100 V within the Mini-Protean system (Bio-Rad, Spain). Following blocking with 4% (w/v) non-fat milk, blots were probed with monospecific antibodies (Salvucci *et al.* 2001). Immunodetection of Rca protein via colorimetry was carried out with the BCIP/NBT alkaline phosphatase system according to the manufacturer's instructions (Sigma-Aldrich Co. LLC., USA). The relative amount of Rubisco activase in each sample was determined by whole-band analysis of the membrane using an image acquisition densitometer (ChemiDoc XRS+ system, Bio-rad, Spain), with the image analysis software Quantity One v4.6.5 (Bio-Rad Laboratories, USA).

Statistical analysis

The statistical significance of trait variation was tested by factorial ANOVA, with species, irrigation treatments and growth temperatures as fixed factors, and the interaction between treatments. Post hoc comparison between treatments was performed using the Duncan test ($P < 0.05$) in the Statistica 6.0 software package (StatStof Inc., USA). Regression coefficients were calculated with the 11.0 Sigma Plot software package (Systat Software Inc., Germany).

RESULTS

Photosynthetic limitations in cereals under water deficit and high temperature stresses

The effects of water deficit (WD) and high growth temperature (HT) on the growth and physiology of rice, wheat and maize were addressed in previous studies (Perdomo *et al*. 2014a, b). The detrimental effects of these two stresses on the gross CO_2 assimilation rate (A_G) and stomatal (g_s) and mesophyll conductance (g_m) are shown in Table S1. These data were used, together with Rubisco activity, to determine the contribution of the different types of limitations to photosynthesis under WD, HT and its combination.

Under WD, the diffusive limitations (D_L) accounted for most of the photosynthetic limitations in wheat, while the biochemical limitations (B_L) were predominant in maize and both type of limitations had a similar contribution in rice (Fig. 1A). Importantly, the analysis of the biochemical limitations in maize was restricted to the C_3 cycle activity, taking into account those limitations associated with Rubisco and not with the C_4 cycle activity of phosphoenolpyruvate carboxylase (PEPC). Under HT, the contribution of B_L was larger than that of D_L and explained most of the inhibition of the photosynthetic CO_2 assimilation in rice and in the C_3-cycle in maize (Fig. 1B). Under the combination of the two stresses (HT-WD), B_L were again more prominent than D_L in rice and in maize, whereas both limitations contributed equally to the inhibition of photosynthesis in wheat (Fig. 1C).

.

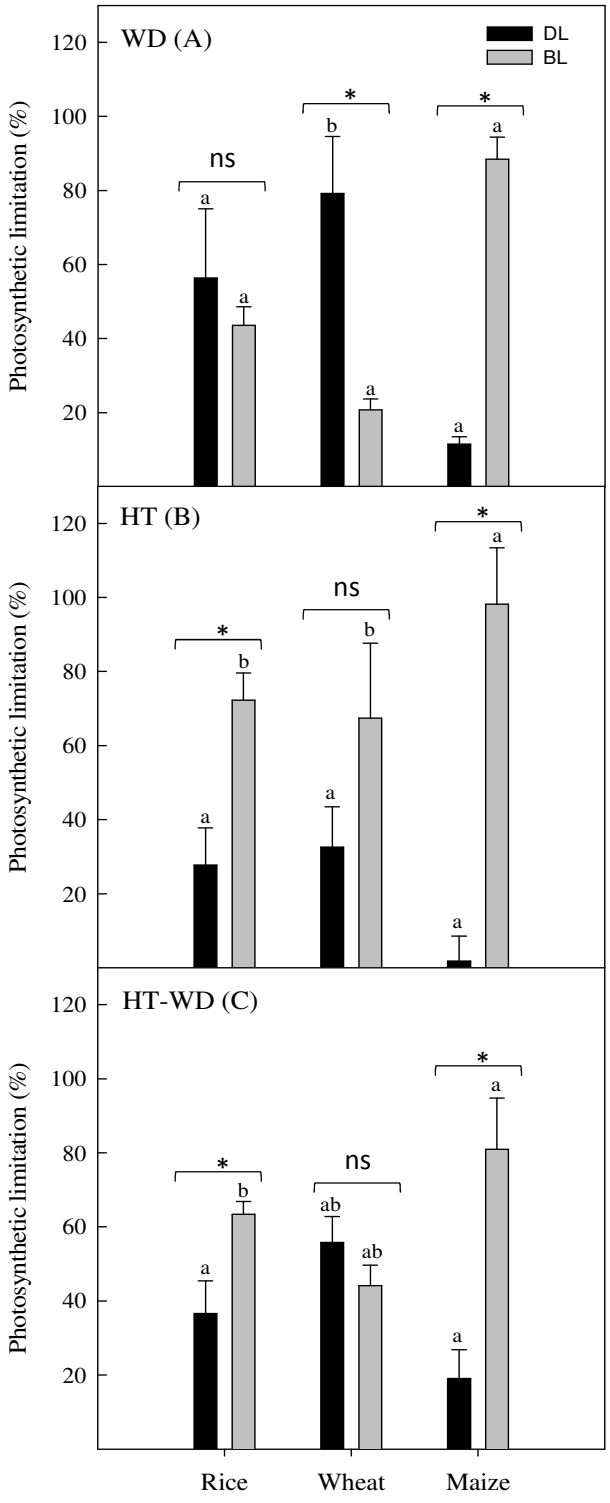

Figure 1. The diffusive (D_L) and biochemical limitations (B_L) to CO_2 assimilation in plants grown under water deficit (WD, A), high temperature (HT, B) and a combination of high temperature and water deficit (HT-WD, C). Values represent means ± SE (n=4–5). Statistically significant differences by Duncan analysis ($P < 0.05$) across the different treatments within each species and limitation are

represented by different letters, and between types of limitation within each species and treatment by asterisks

Table S1. The gross photosynthesis (A_G), the stomatal conductance (g_s) and the mesophyll conductance (g_m) measured at 25ºC in plants grown at 25ºC and well-watered conditions (control), 25ºC and water-deficit conditions (WD), 38ºC and well-watered conditions (HT) and 38ºC and water-deficit conditions (HT-WD). Values are means ± SE (n=4–5). These data was used, together with the *in vitro* Rubisco activity, in the analyses of photosynthetic limitations. For maize, g_m was considered constant across treatments at 2000 mmol m^{-2} s^{-1} (von Caemmerer 2000).

Species	Treatments	A_G (µmol CO_2 m^{-2} s^{-1})	g_s (mmol CO_2 m^{-2} s^{-1})	g_m (mmol CO_2 m^{-2} s^{-1})
Rice	Control	26.6±2.1^{c}	299±32^{b}	439±86^{b}
Rice	WD	11.6±1.5^{a}	57±8^{a}	187±46^{a}
Rice	HT	19.0±0.6^{b}	226±31^{b}	197±13^{a}
Rice	HT-WD	11.4±1.3^{a}	125±21^{a}	107±7^{a}
Wheat	Control	24.2±0.9^{b}	325±19^{b}	198±4^{a}
Wheat	WD	15.4±0.7^{a}	59±5^{a}	664±60^{b}
Wheat	HT	21.5±1.0^{b}	388±34^{b}	143±9^{a}
Wheat	HT-WD	14.4±1.0^{a}	125±21^{a}	106±8^{a}
Maize	Control	27.3±1.9^{c}	103±9^{b}	2000
Maize	WD	19.4±0.4^{b}	62±2^{a}	2000
Maize	HT	12.1±1.4^{a}	73±14^{ab}	2000
Maize	HT-WD	11.2±3.4^{a}	41±11^{a}	2000

The relationship between the net CO_2 assimilation rate (A_N) and the *in vitro* Rubisco activation provided further evidence for the observed photosynthetic limitations. At HT, prevalence of B_L in the three species was confirmed by the positive correlation A_N *vs*. Rubisco activation state in well-watered plants grown at 25ºC or 38ºC and measured at 25ºC (Fig. 2). Maize and rice showed larger decreases in A_N and Rubisco activation state with the increase in temperature (Fig. 2). At WD, the relationship A_N *vs*. Rubisco activation state was positive in rice (R^2 = 0.51, $P < 0.05$, data not shown), but not in wheat and maize ($P > 0.05$, data not shown), in agreement with the limitation analysis (Fig. 1A).

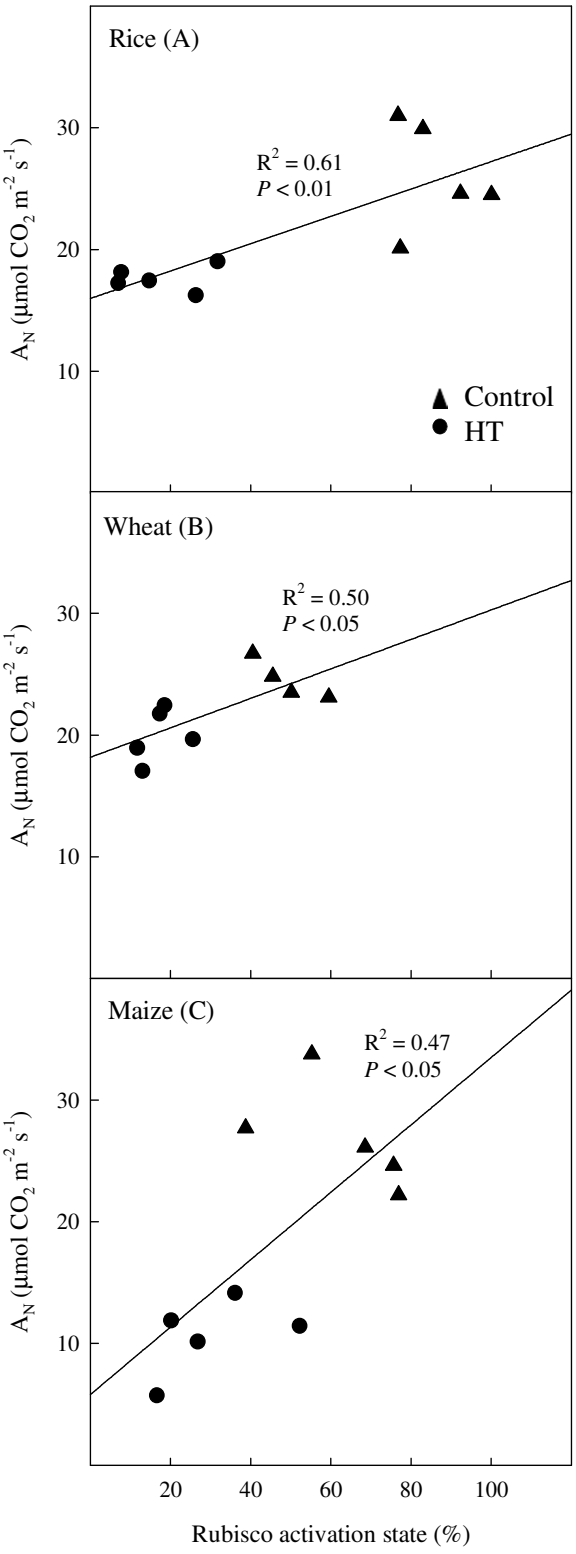

Figure 2. The relationship between the Rubisco activation state and the net CO_2 assimilation rate (A_N) in well-watered plants of rice (A), wheat (B) and maize (C) grown at 25ºC (control) or 38ºC (HT) and measured at 25ºC.

Rubisco amount and activities in cereals under water deficit and high temperature stresses

Water deficit and high temperature stresses affected the amount and activities of Rubisco in rice, wheat and maize differently, depending on the treatment and the species (Fig. 3). Results in Figure 3 are relative to the values obtained for control plants (CT-WW) to facilitate comparison among the three species; however, original values were expressed on a leaf area basis. While the amount of Rubisco in wheat was not affected by any of the applied treatments, it decreased in rice and maize under WD and in rice plants grown at HT (Fig. 3A). The combined HT-WD treatment was not more detrimental than each stress in separate for any of the species; rice was the species with the largest decrease in Rubisco amount, with ca. 50% less Rubisco under HT-WD compared to CT-WW.

Rubisco initial activity was not affected negatively by WD in any of the three species (Fig. 3B). In fact, maize showed an increase in the initial activity, to almost the double under WD compared to the control treatment. By contrast, Rubisco initial activity decreased severely in plants of the three species grown under HT. The combination HT-WD was not more detrimental than HT on its own, which suggests that Rubisco initial activity is more sensitive to inhibition by HT than by WD in these three species. As observed with the amount of Rubisco, rice showed the largest decrease in the initial activity of Rubisco under the combined stress treatment.

Rubisco total activity was less affected than the initial activity under the applied treatments (Fig. 3C). In rice, Rubisco total activity decreased only under HT-WD and non-significant effects were observed in wheat and maize. Overall, the different response between the initial and total activities indicates that the applied treatments affected the Rubisco activation state, particularly under HT and HT-WD (Fig. 3B, C).

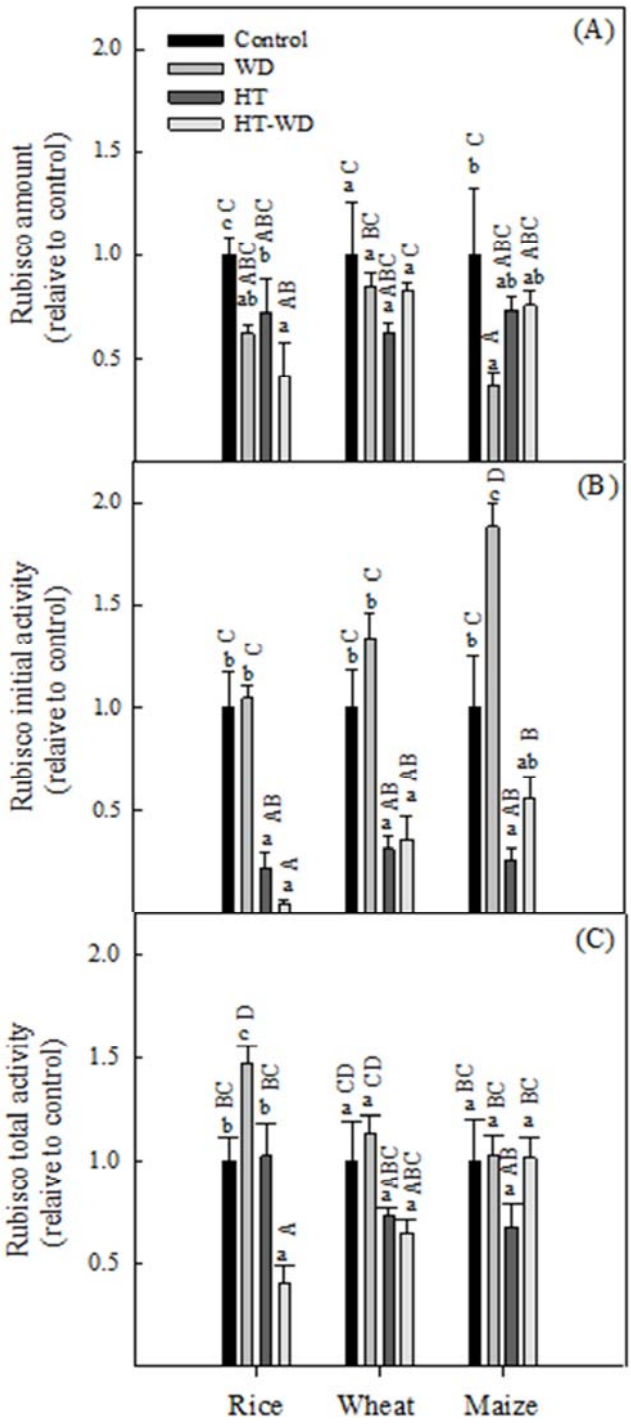

Figure 3. Rubisco amount (A) and initial (B) and total (C) activities at 25°C measured in plants of rice, wheat and maize grown at control, water deficit (WD), high temperature (HT) and a combination of high temperature and water deficit (HT-WD) conditions. To unify scales among the different species, values are means ± SE (n=5) of each parameter expressed relative to control plants. Different letters denote statistically significant differences by Duncan analysis ($P < 0.05$) among species × treatment interactions (uppercase) and between treatments within each species (lowercase).

Rubisco activase amount in cereals under water deficit and high temperature stresses

The total amount of Rubisco activase (Rca) relative to plants grown under control conditions (CT-WW) was not significantly affected by water deficit and high temperatures, except in wheat where Rca increased in plants exposed to the combination HT-WD treatment (Fig. 4A). With the exception of wheat, the Rca amount was constant under the different treatments, which indicates that the decrease in Rubisco activity was not due to a decrease in the total Rca amount. However, when the large and small Rca isoforms were quantified separately some differences among treatments and species became apparent. The large isoform was observed only in the two C_3 species; in rice the amount was slightly higher at HT than HT-WD, whereas in wheat the amount was higher under WD and HT-WD than under HT alone (Fig. 4B). The results suggest that the large isoform is susceptible to HT but induced under WD in wheat. The amount of the small Rca isoform did not show significant differences among the treatments in rice and maize. Conversely, in wheat the amount of the small isoform increased considerably under the combined stresses HT-WD (Fig. 4C).

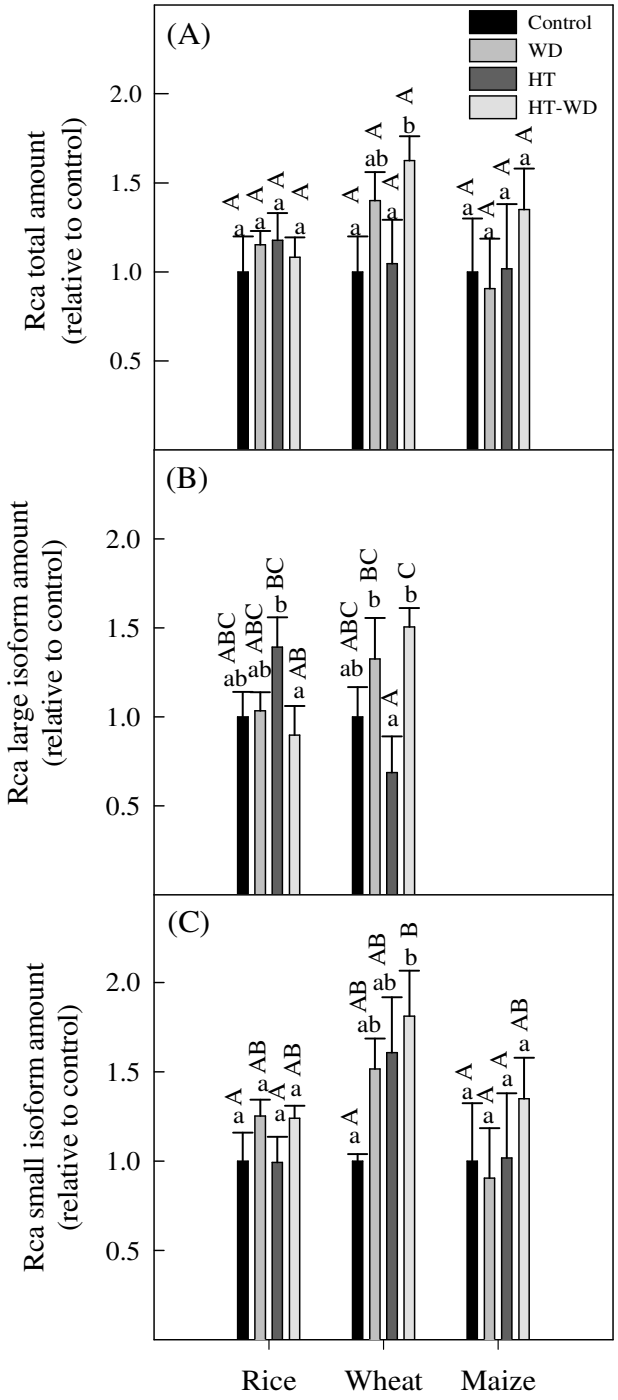

Figure 4. Total Rubisco activase (Rca) amount (A), Rca large subunit amount (B) and Rca small subunit amount (C) in plants of rice, wheat and maize grown at control, water deficit (WD), high temperature (HT) and a combination of high temperature and water deficit (HT-WD) conditions. Values represent means ± SE (n=4–5) of amounts expressed relative to control plants. Different letters denote statistically significant differences by Duncan analysis ($P < 0.05$) among species × treatment interactions (uppercase) and between treatments within each species (lowercase).

Rubisco activation dependence on the CO_2 availability, Rubisco and Rca amounts and rate of electron transport

The activation state of Rubisco was related to the ratio of Rca/Rubisco amounts and to the concentration of CO_2 in the chloroplast of the mesophyll and the bundle sheath cells (C_c and C_s) in the two C_3 species and maize, respectively (Fig. 5). The two C_3 species presented a similar pattern; under water deficit the decreases in the activation state of Rubisco were minor (in rice) or non-existent (in wheat), and were related to moderate increases in the ratio of Rca/Rubisco amounts and to decreases in C_c (Fig. 5A-D). Rice and wheat plants grown under high temperature stress showed large decreases in Rubisco activation state, which related to modest increases in the Rca/Rubisco amounts and no changes in C_c. Maize presented a similar pattern to that observed in the C_3 species, with the exception that in WD plants an increase in the activation state of Rubisco was related to a large increase in the ratio of Rca/Rubisco amounts (Fig. 5E).

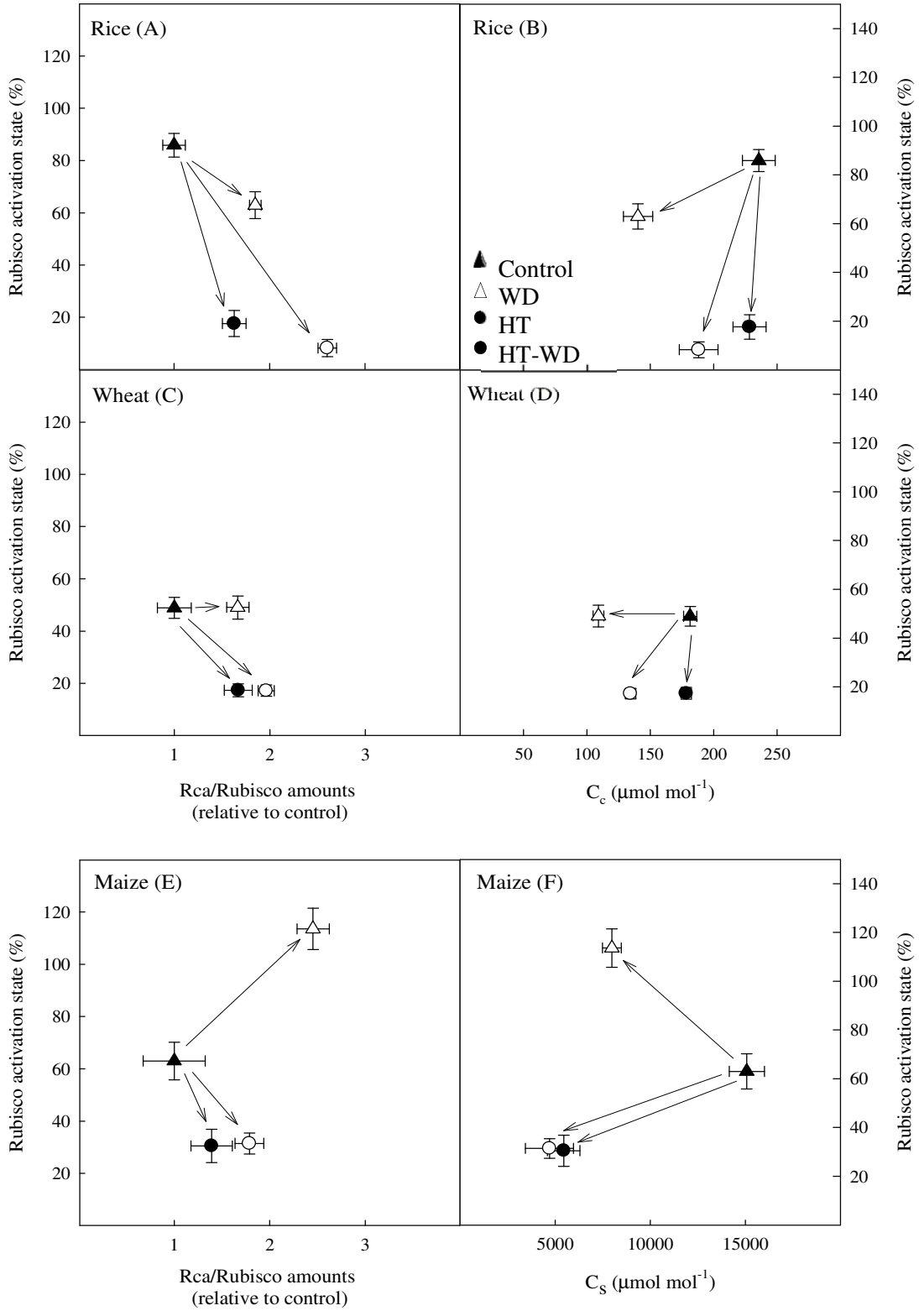

Figure 5. The relationship between the Rubisco activation state and the ratio of Rubisco activase (Rca) to Rubisco amounts (Rca/Rubisco; A, C, E), the CO_2 concentration in the mesophyll chloroplasts (C_c; B, D) or the bundle sheath (C_s; F) in (A, B) rice, (C, D) wheat and (E, F) maize. Values represent means ± SE (n=4–5).

These results do not follow the expected positive relationship between the activation state of Rubisco and the ratio of Rca/Rubisco amounts. Instead, they suggest that changes in the activation of Rubisco are due to the combined effects of adjustments in the ratio of Rca/Rubisco amounts and in C_c or C_s. In fact, increases in the ratio of Rca/Rubisco amounts correlated with decreases in C_c in wheat ($P < 0.05$) and with decreases in C_s in maize ($P < 0.1$) (Fig. 6).

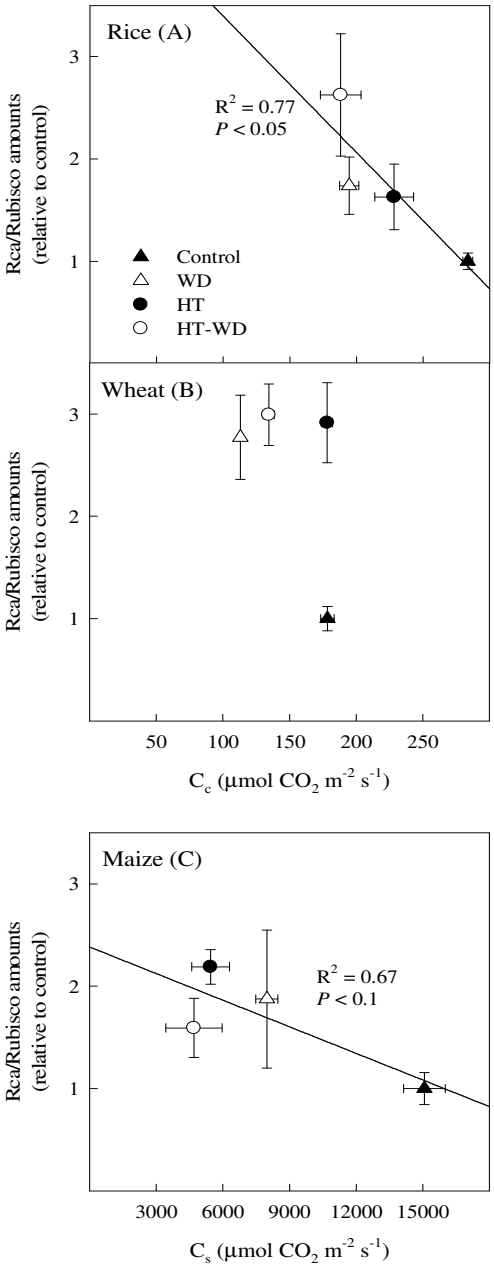

Figure 6. The relationship between the CO_2 concentration in the mesophyll chloroplasts (C_c) in (A) rice and (B) wheat and the CO_2 concentration in the bundle sheath chloroplasts (C_s) in (C) maize and the ratio of Rubisco activase (Rca) to Rubisco amounts (Rca/Rubisco). Values represent means ± SE (n=4–5).

This correlation, which was not observed in wheat, suggests that rice and maize adjusted the ratio of Rca/Rubisco amounts to the concentration of CO_2 available for carboxylation. Rubisco activation state showed a significant positive correlation with the electron transport rate (J) in the two C_3 species (Fig. 7A, B). In rice and wheat, J and Rubisco activation state decreased when the growth temperature increased independently of the irrigation treatment. However, rice showed a slight decrease in J and Rubisco activation state under WD in both growth temperatures, while wheat did not show any differences between WW and WD within each growth temperature. Therefore, rice was the species more affected by the combined HT-WD treatment. Although maize did not show a significant correlation between the Rubisco activation state and J, it showed the same pattern with a decrease in both parameters at HT under WW and WD (Fig. 7C).

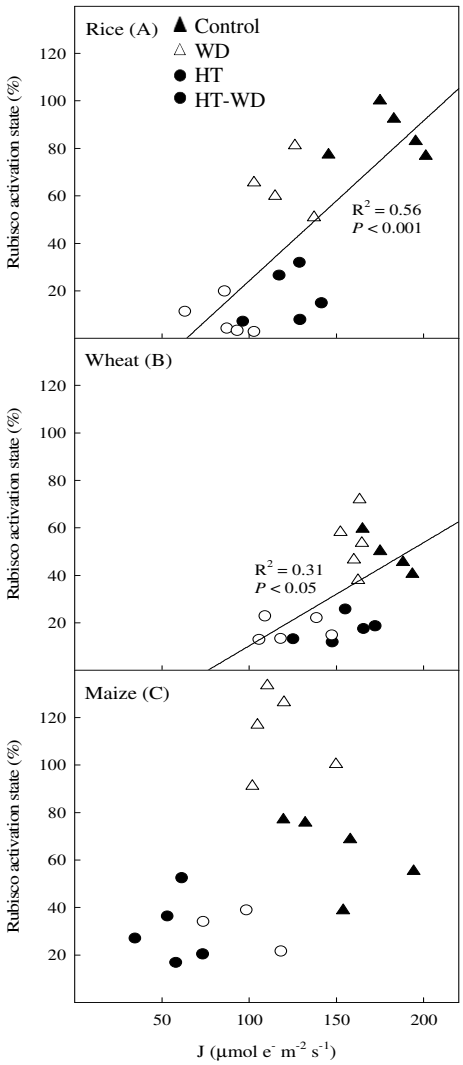

Figure 7. The relationship between the Rubisco activation state and the electron transport rate (J) in (A) rice, (B) wheat and (C) maize.

Discussion

Water deficit and heat stress are two main factors affecting crop productivity. The effects of these stresses, independently and in combination, on the physiological responses of three main cereals, wheat, rice and maize was examined in previous studies (Perdomo *et al.* 2014a, b). In the present manuscript, our focus was on the response of the CO_2-fixing enzyme, Rubisco, and of its molecular chaperone Rubisco activase (Rca). Moreover, physiological and biochemical results were combined to assess the type of limitations to photosynthesis under the two stresses.

Photosynthesis is impaired by diffusion limitations under water deficit and biochemical limitations under high temperature in rice, wheat and maize plants subjected to long-term stressful conditions

The results showed that diffusional limitations (D_L) constrained CO_2 assimilation, at least in the two C_3 species under water deficit, whereas biochemical limitations (B_L) were associated with the inhibition of photosynthesis under heat stress in all three species (Fig. 1), in agreement with previous reports (Chaves *et al.* 2003; Pinheiro & Chaves 2011; Carmo-Silva *et al.* 2012).

Under WD, both C_3 species exhibited reduced stomatal conductance (g_s), and decreases in g_s and mesophyll conductance (g_m) were observed in rice (Table S1). Hence, decreased capacity to transfer CO_2 from the atmosphere to the chloroplast stroma under WD imposed a limitation on photosynthesis in the C_3 species, particularly in wheat (Fig. 1). Decreased g_s and g_m under WD have been shown to limit the CO_2 concentration at the Rubisco site in the mesophyll cells (C_c) of C_3 species and in the bundle-sheet cells (C_s) in C_4 species (Flexas & Medrano 2002; Chaves *et al.* 2003; Ghannoum 2009; Lopes *et al.* 2011). This is confirmed by the present study (Fig. 5B, D, F). In rice, the lower concentration of CO_2 imposed a biochemical limitation by decreasing the activation state of Rubisco (Fig. 5B), which explains the similar contribution of D_L and B_L under WD (Fig. 1A). On the contrary, decreased C_c in wheat under WD did not result in lower Rubisco activation state, which may explain why BL was lower in this species (Fig. 1D). These results suggest that Rubisco in rice is more sensible to de-activation than wheat Rubisco at decreasing CO_2 availability. Different sensibilities of Rubisco de-activation under limiting C_c have been reported among species from contrasting environments (Galmés et al. 2011JXB). Growth at HT did not

alter C_c, but decreased the activation state of Rubisco in rice and wheat (Fig. 5B, D), in agreement with the predominant role of B_L under HT (Fig. 1). A recent report indicates that leaf conductances tended to remain unchanged and/or increase at measuring temperatures around 40ºC in rice and wheat plants grown at optimum temperatures (von Caemmerer & Evans 2014). In our study, no changes were observed in g_s in rice and wheat plants grown at HT and measured at 25ºC, and g_m was decreased only in rice (Table S1).

The analysis of limitations of the C_3 cycle in maize revealed that B_L prevailed both under WD and HT (Fig. 1), suggesting that the observed decrease in C_s under WD was not the main responsible for the lower CO_2 assimilation rates (Table S1 and Fig. 5). Rubisco in maize was markedly affected by WD (decreased amount) and HT (decreased Rubisco activation state) (Fig. 3). This decrease in Rubisco activation state in HT-grown maize was related to important inhibition of the photosynthetic capacity (Fig. 2), as already reported in maize by other authors (Crafts-brandner & Salvucci 2002). These results are in agreement with previous observations that although the C_4 CO_2-concentrating mechanism offers a greater buffering capacity against diffusion limitations under water stress, the biochemistry of C_4 photosynthesis is as sensitive as that of C_3 photosynthesis (Ghannoum 2009).

Rubisco initial activity was also markedly affected in plants of all three species under the combined effect of HT and WD (Fig. 3B), which has been observed in different cotton cultivars (Carmo-Silva *et al.* 2012). In rice and maize, B_L were predominant under the combined treatment whereas in wheat, both D_L and B_L contributed to inhibit photosynthesis when the two stresses occurred in simultaneous (Fig. 1).

Biochemical limitations are mainly attributed to changes in the Rubisco activation state via adjustments in the concentration of CO_2, Rubisco/Rca relative amounts and Rca activity

To understand the effects of water deficit and high temperatures on photosynthesis, it is relevant to elucidate the biochemical components that are affected, particularly those associated with Rubisco. Water deficit effects on Rubisco are still unresolved, with studies showing no effect (Vapaavuori 1986; Pelloux *et al.* 2001) and others reporting decreases in Rubisco content and activation (Flexas *et al.* 2006b; Galmés *et al.* 2011). Some reports show that decreases in the Rubisco content and

activity are due to the severity of water deficit and also are species-specific (Parry *et al.* 2002; Tezara *et al.* 2002; Bota, Medrano & Flexas 2004). In rice and maize, but not in wheat, the amount of Rubisco decreased under WD, but Rubisco initial and total activities increased in maize and rice, respectively (Fig. 3). Other authors have reported a decrease in the initial and total activities of Rubisco that has been attributed to a decrease in the Rubisco content (Flexas & Medrano 2002; Tezara *et al.* 2002; Bota *et al.* 2004; Galmés *et al.* 2013). In the present study, the increased Rubisco activity accompanied by a decrease in the Rubisco content in WD-maize resulted in a higher Rubisco activation state, probably triggered by an increased ratio in the Rca/Rubisco concentration (Fig. 5E).

Several authors have reported that Rubisco amount is highly affected by growth at high temperatures (Verlag *et al.* 2002; Gesch *et al.* 2003; Pérez *et al.* 2011). In the present study, the Rubisco amount was significantly lower at HT only for rice (Fig. 3). However, large decreases in the Rubisco initial activity were observed at HT in all three species, which were not accompanied by changes in the Rubisco total activity. Overall, these data indicate that growing at HT induced a decrease in the Rubisco activation state in the three species. Further, the decrease in the Rubisco activation state provoked a decrease in the photosynthetic capacity of the three crop species (Fig. 2), in agreement with previous reports (Crafts-Brandner & Salvucci 2000; Salvucci & Crafts-brandner 2004; Yamori & von Caemmerer 2009; Scafaro *et al.* 2012). Though, this decrease in the Rubisco activation state at HT was not related to the amount of Rubisco and Rca in any of the three species (Fig. 5A, C, E). This discrepancy may be attributed to the fact that Rubisco activity was measured at 25ºC for both CT- and HT-plants, while Rubisco and Rca amounts are strictly dependent on the growth temperature. However, others have also shown that temperature response of Rubisco activation does not appear to be strongly dependent on Rca content (Salvucci, DeRidder & Portis 2006; Yamori & von Caemmerer 2009). The total Rca amount remained unchanged across treatments in the three species (Fig. 4A), with the exception of wheat where increased Rca amount was observed in the combined treatment HT-WD.

Rca is composed of small and large isoforms (Salvucci *et al.* 1987). Changes in the amount of the large Rca isoform in rice (slight increase) and wheat (slight decrease) at HT did not explain the large decreases in the Rubisco activation state (Figs. 4 and 5). However, decreased Rubisco activation state at HT correlated with the electron transport rate (J) in rice and wheat, irrespective of the watering treatment (Fig. 7). This

correlation did not hold for maize, a species containing only the small isoform of Rca (Fig. 4B). Lower J may result in decreased ATP/ADP ratios and redox potential in the chloroplast, which in turn, could affect the activity of Rca and, consequently, the capacity to restore the activity of Rubisco (Zhang & Portis 1999; Zhang *et al.* 2002; Sage & Kubien 2007). In addition to the limited J in plants grown at HT, Rca activity may be also affected by other processes which have not been measured in the present study and cannot be ruled out. In particular, at high temperatures protons can leak through the thylakoid membrane, impairing the coupling of ATP synthesis to electron transport (Bukhov *et al.* 1999; Bukhov, Samson & Carpenter 2000; Pastenesz & Horton 2014). Furthermore, the results are also consistent with the hypothesis that the intrinsic heat sensitivity of Rca may be linked with the observed decrease in Rubisco activation (Salvucci & Crafts-brandner 2004; Barta *et al.* 2010; Carmo-Silva & Salvucci 2011).

Concluding remarks

Photosynthesis was mainly affected by diffusive limitations under water deficit and by biochemical limitations at high temperature in rice, wheat and maize. Biochemical limitations still predominanted under the combination of WD and HT in rice and maize. Increased biochemical limitations under HT were mainly attributed to decreased Rubisco activation state. In turn, decreased Rubisco activation was not related to altered expression of Rca but correlated with changes in the rate of electron transport. This result suggests that inhibited Rca activity was the main responsible for the observed decrease in the Rubisco activation state, and ultimately, in the photosynthetic CO_2 assimilation. Since changes in Rubisco activity had a direct impact in the photosynthetic capacity of plants, a better understanding of how Rubisco responds to the detrimental factors of climate change is of pivotal importance to predict consequences of future climate on agriculture and natural ecosystems.

Acknowledgements

This study was financed by the contract AGL2009-07999 (Plan Nacional, Spain) awarded to JG. JAP was recipient of a FPI grant from the Govern de les Illes Balears. ECS is supported by the BBSRC 20:20 Wheat Institute Strategic Program (BBSRC BB/J/00426X/1 20:20 Wheat). We thank to Trinidad García (radioisotope service at

UIB) for technical assistance with the radioactive measurements and Miquel Truyols for technical assistance at the greenhouse.

Reference

Aranjuelo I., Perez P., Hernandez L., Irigoyen J.J., Zita G., Martinez-Carrasco R., *et al.* (2005) The response of nodulated alfalfa to water supply, temperature and elevated CO_2: photosynthetic downregulation. *Physiologia Plantarum* **123**, 348–358.

Barta C., Dunkle A.M., Wachter R.M. & Salvucci M.E. (2010) Structural changes associated with the acute thermal instability of Rubisco activase. *Archives of Biochemistry and Biophysics* **499**, 17–25.

Bermúdez M.A., Galmés J., Moreno I., Mullineaux PM., Gotor C. & Romero L.C. (2012) Photosynthetic adaptation to length of day is dependent on S-Sulfocysteine synthase activity in the thylakoid lumen. *Plant Physiology* **160,** 274-288.

Bernacchi C.J., Portis A.R., Nakano H., von Caemmerer S. & Long S.P. (2002) Temperature response of mesophyll conductance. Implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis *in vivo*. *Plant Physiology* **130**, 1992–1998

Bota J., Medrano H. & Flexas J. (2004) Is photosynthesis limited by decreased Rubisco activity and RuBP content under progressive water stress? *New Phytologist* **162**, 671–681.

Bukhov N., Wiese C., Neimanis S. & Heber U. (1999) Heat sensitivity of chloroplasts and leaves: leakage of protons from thylakoids and reversible activation of cyclic electron transport. *Photosynthesis Research* **59**, 81–93.

Bukhov N.G., Samson G. & Carpenter R. (2000) Nonphotosynthetic reduction of the intersystem electron transport chain of chloroplasts following heat stress. *Photochemistry and Photobiology* **72**, 351–357.

von Caemmerer S. (2000) Biochemical models of leaf photosynthesis. CSIRO, Collingwood.

von Caemmerer S. & Evans J.R. (2014) Temperature responses of mesophyll conductance differ greatly between species. *Plant, Cell & Environment.*

Carmo-Silva A.E. & Salvucci M.E. (2013) The regulatory properties of Rubisco activase differ among species and affect photosynthetic induction during light transitions. *Plant Physiology* **161**, 1645–1655.

Carmo-Silva A.E., Gore M.A., Andrade-Sanchez P., French A.N., Hunsaker D.J. & Salvucci M.E. (2012) Decreased CO_2 availability and inactivation of Rubisco limit photosynthesis in cotton plants under heat and drought stress in the field. *Environmental and Experimental Botany* **83**, 1–11.

Carmo-Silva A.E., Powers S.J., Keys A.J., Arrabaça M.C. & Parry M.A.J. (2008) Photorespiration in C_4 grasses remains slow under drought conditions. *Plant, Cell & Environment* **31**, 925–40.

Carmo-Silva A.E. & Salvucci M.E. (2011) The activity of Rubisco's molecular chaperone, Rubisco activase, in leaf extracts. *Photosynthesis Research* **108**, 143–155.

Carmo-Silva A.E., Scales J.C., Madgwick P. & Parry M.A.J. (2014) Optimising Rubisco and its regulation for greater resource use efficiency. *Plant, Cell & Environment*.

Chaves M., Maroco J. & Pereira J. (2003) Understanding plant responses to drought—from genes to the whole plant. *Functional Plant Biology* **30**, 239–264.

Chaves M.M., Flexas J. & Pinheiro C. (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Annals of Botany* **103**, 551–560.

Crafts-Brandner S.J. & Salvucci M.E. (2000) Rubisco activase constrains the photosynthetic potential of leaves at high temperature and CO_2. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 13430–13435.

Crafts-brandner S.J. & Salvucci M.E. (2002) Sensitivity of photosynthesis in a C_4 plant, maize, to heat stress. *Plant Physiology* **129**, 1773–1780

Dore M.H.I. (2005) Climate change and changes in global precipitation patterns: what do we know? *Environment International* **31**, 1167–1181.

Dreyer E., Le Roux X., Montpied P., Daudet F.A. & Masson F. (2001) Temperature response of leaf photosynthetic capacity in seedlings from seven temperate tree species. *Tree Physiology* **21**, 223–32

Evans J.R. & von Caemmerer S. (2013) Temperature response of carbon isotope discrimination and mesophyll conductance in tobacco. *Plant, Cell & Environment* **36**, 745–756

Flexas J., Bota J., Galmés J., Medrano H. & Ribas-Carbó M. (2006a) Keeping a positive carbon balance under adverse conditions: responses of photosynthesis and respiration to water stress. *Physiologia Plantarum* **127**, 343–352.

Flexas J., Bota J., Loreto F., Cornic G. & Sharkey T.D. (2004) Diffusive and metabolic limitations to photosynthesis under drought and salinity in C_3 plants. *Plant Biology* **6**, 269–79.

Flexas J., Escalona J.M. & Medrano H. (1999) Water stress induces different levels of photosynthesis and electron transport rate regulation in grapevines. *Plant, Cell and Environment* **22**, 39–48.

Flexas J. & Medrano H. (2002) Drought-inhibition of photosynthesis in C_3 plants: stomatal and nonstomatal limitations revisited. *Annals of Botany* **89**, 183–189.

Flexas J., Ribas-Carbó M., Bota J., Galmés J., Henkle M., Martínez-Cañellas S., *et al.* (2006b) Decreased Rubisco activity during water stress is not induced by decreased relative water content but related to conditions of low stomatal conductance and chloroplast CO_2 concentration. *New Phytologist* **172**, 73–82.

Flexas J., Ribas-Carbó M., Diaz-Espejo A., Galmés J. & Medrano H. (2008) Mesophyll conductance to CO_2: current knowledge and future prospects. *Plant, Cell & Environment* **31**, 602–21.

Galmés J., Abadía A., Cifre J., Medrano H. & Flexas J. (2007a) Photoprotection processes under water stress and recovery in Mediterranean plants with different growth forms and leaf habits. *Physiologia Plantarum* **130**, 495–510.

Galmés J., Aranjuelo I., Medrano H. & Flexas J. (2013) Variation in Rubisco content and activity under variable climatic factors. *Photosynthesis Research* **117**, 73–90.

Galmés J., Medrano H. & Flexas J. (2007b) Photosynthetic limitations in response to water stress and recovery in Mediterranean plants with different growth forms. *New Phytologist* **175**, 81–93.

Galmés J., Ribas-Carbó M., Medrano H. & Flexas J. (2011) Rubisco activity in Mediterranean species is regulated by the chloroplastic CO_2 concentration under water stress. *Journal of Experimental Botany* **62**, 653–665.

Genty B., Briantais J. & Baker N. (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* **990**, 87–92.

Gesch R.W., Kang I., Vu J.C.V, Boote K.J., Allen L.H. & Bowes G. (2003) Rubisco expression in rice leaves is related to genotypic variation of photosynthesis under elevated growth CO_2. *Plant, Cell and Environment* **26**, 1941–1950.

Ghannoum O. (2009) C_4 photosynthesis and water stress. *Annals of Botany* **103**, 635–644.

Gunderson C., Norby R. & Wullschleger S. (2000) Acclimation of photosynthesis and respiration to simulated climatic warming in northern and southern populations of *Acer saccharum*: laboratory and field evidence. *Tree Physiology* **20**, 87–96.

Harley P.C., Loreto F., Di Marco G. & Sharkey T.D. (1992) Theoretical considerations when estimating the mesophyll conductance to CO_2 flux by analysis of the response of photosynthesis to CO_2. *Plant Physiology* **98**, 1429–36.

IPCC (2013) *Working Group I Contribution to the IPCC Fifth Assessment Report Climate Change 2013: The Physical Science Basis Summary for Policymakers*.

Kim K. & Portis A.R. (2005) Temperature dependence of photosynthesis in Arabidopsis plants with modifications in Rubisco activase and membrane fluidity. *Plant & Cell Physiology* **46**, 522–530.

Krall J. & Edwards G. (1992) Relationship between photosystem II activity and CO_2 fixation in leaves. *Physiologia Plantarum* **86**, 180–187.

Laisk A. & Loreto F. (1996) Determining photosynthetic parameters from leaf CO_2 exchange and chlorophyll fluorescence. *Plant Physiology* **110**, 903–912.

Lobell D. & Asner G. (2003) Climate and management contributions to recent trends in U.S. agricultural yields. *Science* **299**, 1032.

Lopes M.S., Araus J.L., van Heerden P.D.R. & Foyer C.H. (2011) Enhancing drought tolerance in C_4 crops. *Journal of Experimental Botany* **62**, 3135–3153.

Loriaux S.D., Avenson T.J., Welles J.M., McDermitt D.K., Eckles R.D., Riensche B., *et al.* (2013) Closing in on maximum yield of chlorophyll fluorescence using a single multiphase flash of subsaturating intensity. *Plant, Cell & Environment* **36**, 1755–1770.

Makino A. & Sage R.F. (2007) Temperature response of photosynthesis in transgenic rice transformed with “sense” or “antisense” *rbc*S. *Plant & Cell Physiology* **48**, 1472–1483.

Massad R.S., Tuzet A. & Bethenod O. (2007) The effect of temperature on C_4-type leaf photosynthesis parameters. *Plant, Cell & Environment* **30**, 1191–1204.

Mathur S., Agrawal D. & Jajoo A. (2014) Photosynthesis: Response to high temperature stress. *Journal of Photochemistry and Photobiology. B, Biology* **137**, 116–126.

Mott K.A. & Woodrow I.E. (2000) Modelling the role of Rubisco activase in limiting non-steady-state photosynthesis. *Jounal of Experimental Botany* **51**, 399–406.

Panković D., Sakac Z., Kevresan S. & Plesnicar M. (1999) Acclimation to long-term water deficit in the leaves of two sunflower hybrids: photosynthesis, electron transport and carbon metabolism. *Journal of Experimental Botany* **50**, 127–138.

Parry M.A.J., Andralojc P., Khan S., Lea P. & Keys A. (2002) Rubisco activity: Effects of drought stress. *Annals of Botany* **89**, 833–839.

Pastenesz C. & Horton P. (2014) Effect of High Temperature on photosynthesis in beans. *Plant Physiology* **112**, 1245–1251.

Pelloux J., Jolivet Y., Fontaine V., Banvoy J. & Dizengremel P. (2001) Changes in Rubisco and Rubisco activase gene expression and polypeptide content in *Pinus halepensis* M. subjected to ozone and drought. *Plant, Cell & Environment* **24**, 123–131.

Perdomo J.A., Conesa M.À., Medrano H., Ribas-Carbó M. & Galmés J. (2014a) Effects of long-term individual and combined water and temperature stress on the growth of rice, wheat and maize: relationship with morphological and physiological acclimation. *Physiologia Plantarum.*

Perdomo J.A., Carmo-Silva A.E., Hermida C., Flexas J. & Galmés J. (2014b) Biochemical and diffusive limitations to photosynthesis in rice, wheat and maize grown at high temperature and under water deficit. *In preparation*.

Pérez P., Alonso A., Zita G., Morcuende R. & Martínez-Carrasco R. (2011) Down-regulation of Rubisco activity under combined increases of CO_2 and temperature minimized by changes in Rubisco k_{cat} in wheat. *Plant Growth Regulation* **65**, 439–447.

Pinheiro C. & Chaves M.M. (2011) Photosynthesis and drought: can we make metabolic connections from available data? *Journal of Experimental Botany* **62**, 869–882.

Robinson S. & Portis A. (1989) Ribulose-1,5-bisphosphate carboxylase/oxygenase activase protein prevents the *in vitro* decline in activity of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Plant Physiology* **90**, 968–971.

Sage R.F. & Kubien D.S. (2007) The temperature response of C_3 and C_4 photosynthesis. *Plant, Cell & Environment* **30**, 1086–1106.

Salvucci M.E., Portis A.J. & Ogren W. (1985) A soluble chloroplast protein catalyzes ribulosebisphosphate carboxylase/oxygenase activation *in vivo*. *Photosynthesis Research*, **7,** 193–201.

Salvucci M.E., Werneke J., Ogren W. & Portis A.J. (1987) Purification and species distribution of Rubisco activase. *Plant Physiology* **84**, 930-936.

Salvucci M.E. & Crafts-brandner S.J. (2004) Relationship between the heat tolerance of photosynthesis and the thermal stability of Rubisco activase in plants from contrasting thermal environments. *Plant Physiology* **134**, 1460–1470.

Salvucci M.E., DeRidder B.P. & Portis A.R.J. (2006) Effect of activase level and isoform on the thermotolerance of photosynthesis in Arabidopsis. *Journal of Experimental Botany* **57**, 3793–3799.

Salvucci M.E., Osteryoung K.W., Crafts-brandner S.J. & Vierling E. (2001) Exceptional sensitivity of Rubisco activase to thermal denaturation *in vitro* and *in vivo*. *Plant Physiology* **127**, 1053–1064.

Scafaro A.P., Yamori W., Carmo-Silva A.E., Salvucci M.E., von Caemmerer S. & Atwell B.J. (2012) Rubisco activity is associated with photosynthetic thermotolerance in a wild rice (*Oryza meridionalis*). *Physiologia Plantarum* **146**, 99–109.

Scales J.C., Parry M.A.J. & Salvucci M.E. (2014) A non-radioactive method for measuring Rubisco activase activity in the presence of variable ATP:ADP ratios, including modifications for measuring the activity and activation state of Rubisco. *Photosynthesis Research* **119**, 355–365.

Schrader S.M., Wise R.R., Wacholtz W.F., Ort D.R. & Sharkey T.D. (2004) Thylakoid membrane responses to moderately high leaf temperature in Pima cotton. *Plant, Cell and Environment* **27**, 725–735.

Schultz H. (1996) Leaf absorptance of visible radiation in *Vitis vinifera* L.: estimates of age and shade effects with a simple field method. *Scientia Horticulturae* **66**, 93–102.

Spreitzer R.J. & Salvucci M.E. (2002) Rubisco: structure, regulatory interactions, and possibilities for a better enzyme. *Annual Review of Plant Biology* **53**, 449–475.

Tezara W., Mitchell V., Driscoll S.P. & Lawlor D.. (2002) Effects of water deficit and its interaction with CO_2 supply on the biochemistry and physiology of photosynthesis in sunflower. *Journal of Experimental Botany* **53**, 1781–1791.

Tezara W., Mitchell V.J., Driscoll S.D. & Lawlor D.W. (1999) Water stress inhibits plant photosynthesis by decreasing coupling factor and ATP. *Letters to Nature* **401**, 914–917.

Tosens T., Niinemets Ü., Vislap V., Eichelmann H. & Castro Díez P. (2012) Developmental changes in mesophyll diffusion conductance and photosynthetic capacity under different light and water availabilities in *Populus tremula*: how structure constrains function. *Plant, Cell & Environment* **35**, 839–856.

Vapaavuori E. (1986) Correlation of activity and amount of ribulose 1,5-bisphosphate carboxylase with chloroplast stroma crystals in water-stressed willow leaves. *Journal of Experimental Botany* **37**, 89–98.

Verlag F., Vu J.C.V, Newman Y.C., Allen L.H., Gallo-meagher M. & Zhang M. (2002) Photosynthetic acclimation of young sweet orange trees to elevated growth CO_2 and temperature. *Journal of Plant Physiology* **159**, 147–157.

Vile D., Pervent M., Belluau M., Vasseur F., Bresson J., Muller B., *et al.* (2012) Arabidopsis growth under prolonged high temperature and water deficit: independent or interactive effects? *Plant, Cell & Environment* **35**, 702–18.

Walker B., Ariza L.S., Kaines S., Badger M.R. & Cousins A.B. (2013) Temperature response of *in vivo* Rubisco kinetics and mesophyll conductance in *Arabidopsis thaliana*: comparisons to *Nicotiana tabacum*. *Plant, Cell & Environment* **36**, 2108–2119.

Way D.A. & Yamori W. (2014) Thermal acclimation of photosynthesis: on the importance of adjusting our definitions and accounting for thermal acclimation of respiration. *Photosynthesis Research* **119**, 89–100.

Yamori W. & von Caemmerer S. (2009) Effect of Rubisco activase deficiency on the temperature response of CO_2 assimilation rate and Rubisco activation state: insights from transgenic tobacco with reduced amounts of Rubisco activase. *Plant Physiology* **151**, 2073–2082.

Yamori W., Hikosaka K. & Way D.A (2014) Temperature response of photosynthesis in C_3, C_4, and CAM plants: temperature acclimation and temperature adaptation. *Photosynthesis Research* **119**, 101–117.

Yamori W., Noguchi K., Kashino Y. & Terashima I. (2008) The role of electron transport in determining the temperature dependence of the photosynthetic rate in spinach leaves grown at contrasting temperatures. *Plant & Cell Physiology* **49**, 583–591.

Yamori W., Suzuki K., Noguchi K., Nakai M. & Terashima I. (2006) Effects of Rubisco kinetics and Rubisco activation state on the temperature dependence of the

photosynthetic rate in spinach leaves from contrasting growth temperatures. *Plant, Cell and Environment* **29**, 1659–1670

Zhang N., Kallis R., Ewy R. & Portis A.R. (2002) Light modulation of Rubisco in Arabidopsis requires a capacity for redox regulation of the larger Rubisco activase isoform. *Proceedings of the National Academy of Sciences* **99**, 3330–3334.

Zhang N. & Portis A.R. (1999) Mechanism of light regulation of Rubisco: A specific role for the larger Rubisco activase isoform involving reductive activation by thioredoxin-f. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 9438–9443.

Zhou Y., Lam H.M. & Zhang J. (2007) Inhibition of photosynthesis and energy dissipation induced by water and high light stresses in rice. Journal of Experimental Botany **58**, 1207–1217.

Chapter 6

TEMPERATURE DEPENDENCE OF RUBISCO KINETICS IN RELATED SPECIES OF *FLAVERIA*

6.1. TEMPERATURE DEPENDENCE OF *IN VITRO* RUBISCO KINETICS IN SPECIES OF *FLAVERIA* WITH DIFFERENT PHOTOSYNTHETIC MECHANISMS

Juan Alejandro Perdomo[1], Amanda P. Cavanagh[2], David S. Kubien[2], Jeroni Galmés[1]*

[1]*Research Group on Plant Biology under Mediterranean Conditions. Universitat de les Illes Balears, Balearic Islands, Spain.*

[2]*Department of Biology, University of New Brunswick, Fredericton, Canada.*

***Corresponding author:** Jeroni Galmés

e-mail: jeroni.galmes@uib.cat

fax: +34 / 971 / 173184

tel: +34 / 971 / 252710

Running title: Temperature response of Rubisco kinetics in *Flaveria*

Abstract

There is general consensus in the literature that different photosynthetic mechanisms (i.e., C_3 vs. C_4) present relevant differences in the kinetic parameters of Rubisco. However, potential differences in the temperature dependencies of Rubisco kinetic parameters between C_3 and C_4 plants are uncertain. With this aim, six species of *Flaveria* with contrasting photosynthetic mechanism (C_3, C_3/C_4 and C_4) were selected and their Rubisco Michaelis-Menten constants for CO_2 and RuBP (K_c and K_{RuBP}), carboxylase catalytic turnover rate (k_{cat}^c) and CO_2/O_2 specificity factor ($S_{c/o}$) measured between 10ºC and 40ºC. The results confirmed different Rubisco catalysis between C_3 and C_4 plants. Rubisco from the C_3 species presented higher E_a for K_c and k_{cat}^c than that from C_4 species, which were translated into differences in the temperature response of the carboxylase catalytic efficiency (k_{cat}^c/K_c). However, E_a did not differ for $S_{c/o}$ or K_{RuBP}. Analysis of the amino acid sequences of the Rubisco large subunit (LSu) confirms the role of Met-309-Ile substitution as the C_4 catalytic switch in *Flaveria* Rubisco, and suggests that this substitution may be also responsible for the contrasting temperature dependence of Rubisco kinetics between C_3 and C_4 *Flaveria* species. Overall, the present study provides the first evidence that the evolutionary adjustment in the temperature sensitivity of Rubisco kinetic properties not only respond to the thermal environment to which the species is adapted (Sage 2002; Galmés et al. 2005), but also depend on the species photosynthetic mechanism.

Keywords:

C_3; C_4; *Flaveria*; kinetics; photosynthesis; Rubisco; temperature.

Introduction

An increase in the global mean temperature is one of the main consequences of anthropogenic carbon emissions; models predict increases of up to 5ºC by 2100, accompanied by more frequent and intense heat waves (IPCC 2013). Such an increase in temperature constitutes a serious challenge to agriculture by decreasing yields in many important crop-growing regions of the world. Among other negative effects, temperature stress reduces crop yields because photosynthesis rates decline above the thermal optimum (Ainsworth and Ort 2010; Berry and Björkman 1980). Stomatal and mesophyll leaf conductances to CO_2 tend to increase with temperature across the physiologically relevant range of temperatures (Bunce 2000; Flexas et al. 2008). Therefore, at elevated temperatures, diffusion of CO_2 to the site of fixation is not limiting increases in photosynthesis, but instead biochemical constraints limit the rate of CO_2 uptake (Sage and Kubien 2007).

Rubisco (ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase) catalyzes the first irreversible enzymatic step of photosynthesis, the addition of CO_2 to RuBP. Rubisco activity is highly responsive to temperature changes (Sharkey 2005; Hikosaka et al. 2006; Yamori et al. 2006; Sage and Kubien 2007), and thus influences the temperature response of net photosynthesis (Yamori et al. 2006; Galmés et al. 2013). Rubisco's maximum carboxylase turnover rate (k_{cat}^c) increases exponentially with temperature (Sage 2002). However, at temperatures higher than the photosynthetic thermal optimum, the increases in k_{cat}^c are not translated into increased CO_2 fixation because of the lower CO_2 affinity of Rubisco, i.e., higher Michaelis-Menten constant for CO_2 (K_c) and lower CO_2/O_2 specificity factor ($S_{c/o}$), and the decreased CO_2/O_2 concentration ratio in solution (Hall and Keys 1983; Jordan and Ogren 1984). Rubisco $S_{c/o}$ decreases with increasing temperature because the enzyme's affinity for O_2 (i.e., $1/K_o$) declines less with increasing temperature than does the affinity for CO_2 (i.e., $1/K_c$) (Andrews and Lorimer 1987). In C_3 plants, these changes favour the oxygenation of RuBP by Rubisco over carboxylation, increasing the flux through photorespiration and ultimately reducing the potential growth at high temperatures (Jordan and Ogren 1984).

The existence of a biochemical CO_2-concentrating mechanism (CCM) in C_4 plants allows Rubisco to operate at CO_2 concentrations several times above ambient levels. Under these conditions, the maximum rates of CO_2 assimilation are primarily determined by k_{cat}^c (Kubien et al. 2003). High CO_2 conditions favour Rubiscos with

faster turnover rates, but this comes at the expense of lower CO_2 affinity; Rubiscos from C_4 plants have higher k_{cat}^c and K_c than the enzyme from C_3 species (Kubien et al. 2008). The structural basis for the catalytic differences between C_3 and C_4 Rubiscos has received recent attention (Christin et al. 2008; Kapralov et al. 2011, 2012; Whitney et al. 2011). In *Flaveria*, a Met-309-Ile substitution in the Rubisco large subunit (LSu), explains the catalytic difference between C_3 and C_4 Rubiscos (Kapralov et al. 2011; Whitney et al. 2011). However, the exploration of the differences in the kinetic parameters of Rubisco from C_3 and C_4 species, and the role of residues 149 and 309, has been limited at the standard temperature of 25ºC, and it is therefore uncertain whether these catalytic differences are maintained at other temperatures.

The temperature response of C_4 Rubisco kinetic parameters has been examined in only two reports. Jordan and Ogren (1984) did not find different temperature responses of $S_{c/o}$ between Rubisco from *Spinacea oleracea* (C_3) and *Amaranthus hybridus* (C_4). Similarly, Sage (2002) did not observe different activation energies for k_{cat}^c between a group of C_4 and C_3 species. Conversely, the temperature response of C_3 Rubisco kinetic parameters has been characterized in more detail, with some reports confirming the existence of interspecific differences in the temperature dependence of k_{cat}^c (Tieszen and Sigurdson 1973; Weber et al. 1977; Zhu et al. 1998; Sage 2002) and $S_{c/o}$ (Zhu et al. 1998; Galmés et al. 2005). Differences in the temperature response of k_{cat}^c and $S_{c/o}$ were ascribed to the thermal conditions typically encountered by the species in their native habitat (Sage 2002; Galmés et al. 2005). C_3 and C_4 photosynthetic mechanisms present different temperature optima (Berry and Björkman 1980; Yamori et al. 2014); it might be important to discern whether C_3 and C_4 Rubiscos are particularly suited for the low and high temperature range, respectively.

The objective of the present study was to discern whether closely related species with different photosynthetic mechanism have Rubiscos with different temperature dependence. With this aim, we characterized and compared the *in vitro* temperature response of the Rubisco CO_2/O_2 specificity ($S_{c/o}$), the Michaelis-Menten constants for CO_2 (K_c) and RuBP (K_{RuBP}) and the maximum carboxylase turnover rate (k_{cat}^c) from *Flaveria* species with C_3 (*F. pringlei* and *F. cronquistii*), intermediate C_3/C_4 (*F. angustifolia* and *F. floridana*) and C_4 photosynthetic mechanisms (*F. bidentis* and *F. trinervia*).

Material and methods

Plant material and growth conditions

Six *Flaveria* species with different photosynthetic mechanisms [*F. pringlei* Gand. and *F. cronquistii* A.M. Powell (C_3), *F. angustifolia* (Cav.) Pers. and *F. floridana* J.R. Johnst. (C_3/C_4), *F. bidentis* (L.) Kuntze and *F. trinervia* (Spreng.) C. Mohr (C_4)] were obtained from the same collection used by (Kubien et al. 2008). Plants were grown in 10 L pots containing 80/20% (v/v) Promix (Plant Products, Brampton, Canada) and sand, in naturally illuminated glasshouses at the University of New Brunswick (45º56' N, 66º33' W), with daytime and night-time air temperatures of 27–33ºC/13–15ºC, respectively. Plants were watered as needed and fertilized biweekly with full-strength Hoagland's solution.

Rubisco extraction

Leaves were harvested 4-5 months after plants were initiated from cuttings. Three leaf discs (4 cm^2) were taken from five plants of each species at midday and frozen immediately in liquid N_2. Rubisco extraction was based on procedures described by (Kubien et al. 2011). Leaf tissue was ground in a cold mortar containing 5 mL of ice-cold extraction buffer consisting in 100 mM Hepes-KOH (pH 7.6), 2 mM EDTA, 5 mM $MgCl_2$ and 4% (v/v) protease inhibitor cocktail (P9599, Sigma, Canada). The homogenate was centrifuged for 5 min at 16,000 g and 4ºC, and the resulting supernatant was concentrated (Amicon 50K spin filters, Millipore, Billerica, USA). Finally, the extracts were made to 20% glycerol (v/v) and stored at -80ºC.

Rubisco CO_2/O_2 specificity factor assay

The concentrated Rubisco extracts were used to measure the CO_2/O_2 specificity factor ($S_{c/o}$) at five temperatures (10ºC, 20ºC, 25ºC, 30ºC and 40ºC), following the method described by Kane et al. (1994). Rubisco $S_{c/o}$ was measured in humidified gas (0.1% CO_2 in O_2) with a total flow of 2000 mL min^{-1} (G400 gas-mixing system, Qubit Systems, Kingston, Canada). The reaction was initiated by the injection of 2 nmol 1-^{3}H-RuBP (3 kBq $nmol^{-1}$), and was terminated after 60 min by the addition of alkaline phosphatase. The ^{3}H-glycerate and ^{3}H-glycolate were separated by high performance liquid chromatography (HPLC) using an Aminex HPX-87H ion-exchange column (Bio-Rad, Canada) maintained at 60°C (Kane et al. 1994), with a flow rate of 0.4 mL min^{-1}.

The HPLC system is described by Shay and Kubien (2013). Glycerate and glycolate fractions were collected in drop synchronization mode (Fraction Collector III, Waters, Milford, USA) and the amount of ^3H in each fraction was determined using a liquid scintillation counter (LS-6500, Beckman Coulter, USA). Rubisco $S_{c/o}$ was calculated from the ratio of ^3H-glycerate to ^3H-glycolate and the mole fractions of CO_2 and O_2, giving a value expressed as a ratio of partial pressures (Kane et al. 1994). To express this parameter in terms of concentrations of the dissolved gases in solution, the parameter was divided by the ratio of aqueous solubility of O_2 and CO_2 (Table S1; Sander 1999).

Table S1. Temperature responses of aqueous solubilities for CO_2 and O_2. The solubility constants at 25°C are taken from Sander (1999) and temperature adjustment was made using the formula:

$$sol(T) = sol(25) \times \exp\left(\frac{\Delta_{soln}H}{R}\left(\frac{1}{T_K}\right) - \left(\frac{1}{298.15}\right)\right)$$

where R is the gas constant (8.314 J $mol^{-1}K^{-1}$), T_K is the temperature in Kelvin, and $\Delta_{soln}H$ is the enthalpy of solution (19.95 kJ mol^{-1} for CO_2 and 14.13 kJ mol^{-1} for O_2).

Temperature (°C)	**CO_2 Solubility (M bar^{-1})**	**O_2 solubility (M bar^{-1})**
10	0.0514	0.00167
20	0.0385	0.00139
25	0.0334	0.00128
30	0.0294	0.00118
40	0.0228	0.00101

Determination of the Rubisco maximum carboxylation rate (k_{cat}^c)

Rubisco k_{cat}^c was determined on fresh leaf extracts, following the procedure described by Kubien et al. (2011). Two leaf disks (1.8 cm^2) were taken in full sunlight, frozen in liquid N_2, and stored at –80ºC until being assayed (< 48 h). Samples were ground in a cold mortar with 3 mL of extraction buffer consisting of 100 mM HEPES-KOH (pH 8.0), 5 mM DTT, 12 mM β-aminocaproic acid, 2.4 mM benzamidine, 10 mg mL^{-1} PVPP, 2 mg mL^{-1} BSA, 2 mg mL^{-1} PEG, 2% (v/v) Tween-80 and 2 mM NaH_2PO_4.

The homogenate was centrifuged for 1 min at 16,000 g and 4ºC, and 900 µL of the supernatant was added to 100 µL of an activating solution (100 mM Bicine–NaOH pH 8.2, 200 mM $MgCl_2$, 10 mM EDTA, and 100 mM $NaHCO_3$), and the mixture was incubated at 25ºC for 30 min to fully carbamylate Rubisco. The activity of the Rubisco enzyme was determined at the same temperatures than $S_{c/o}$ by the incorporation of ^{14}C into acid-stable products, as described by Kubien et al. (2011). Assays were initiated by the addition of 50 µL of activated extract to 250 µL assay buffer (100 mM Bicine-NaOH (pH 8.2), 1 mM Na-EDTA, 20 mM $MgCl_2$, 5 mM DTT, 500 µM RuBP and 12 mM $NaH^{14}CO_3$ [~700 Bq $nmol^{-1}$]), and stopped after 60 s by adding 250 µL of 1 M formic acid. Samples were dried at 90°C and ^{14}C was determined by liquid scintillation counting. The concentration of Rubisco catalytic sites was determined by the $^{14}CABP$ binding assay (Ruuska et al. 1998), assuming eight binding sites per Rubisco (Kubien et al. 2011).

Determination of the Rubisco Michaelis-Menten constants for RuBP and CO_2

The Rubisco Michaelis-Menten constants for RuBP (K_{RuBP}) and CO_2 (K_c) were determined at the same temperatures than $S_{c/o}$. K_{RuBP} was measured as described by Paul et al. (1991) using RuBP synthesized as described by Kane et al. (1998). Seven RuBP concentrations (0 – 200 µM) were used to measure K_{RuBP} in buffer containing 10 mM $NaH^{14}CO_3$. The concentrated Rubisco extracts were diluted to 1 µM catalytic sites with assay buffer (100 mM Bicine (pH 8.2), 200 mM $MgCl_2$, 1 mM EDTA and 10 mM $NaH^{12}CO_3$), and activated for 30 min at 30ºC before adding to the respective RuBP buffer to initiate the assays (final assay volume 250 µL). After 60 s the assays were terminated with 250 µL 1 M formic acid.

To determine K_c, soluble protein was extracted by grinding leaf samples in a mortar with 2 mL of ice-cold extraction buffer consisting of 100 mM Bicine-NaOH (pH 8.2), 0.1 mM EDTA, 60 mg mL^{-1} PEG4000, 10 mM DTT, 50 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM benzamidine, 1 mM □-aminocaproic acid, 2 µM pepstatin, 10 µM E-64 (Sigma, USA) 10 µM chymostatin, 2 mM phenylmethylsulfonyl fluoride and 25 mg mL^{-1} PVP. The homogenate was centrifuged for 4 min at 13,000 g and 4ºC. Low molecular weight proteins and salts present in the leaf crude extracts were removed by passage of supernatant (1 mL) through a PD-10 desalting column, containing Sephadex G-25 (GE Healthcare, USA) column (8.3 mL bed volume) pre-equilibrated and eluted with desalting buffer (100 mM Bicine-NaOH (pH 8.2), 0.5 mM

EDTA, 1 mM KH_2PO_4, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 10 mM DTT, 1 mM benzamidine and 1 mM β-aminocaproic acid). The protein peak (in 1 mL) was supplemented with protease inhibitors (4 μM pepstain A, 20 μM E64 and 20 μM chymostatin) and 250 μL of this mixture was supplemented with sufficient carrier-free $NaH^{14}CO_3$ to adjust the specific radioactivity to 3.7 x 10^{10} Bq mol^{-1}.

Rates of Rubisco $^{14}CO_2$-fixation using the activated protein extract were measured in 7 mL septum capped scintillation vials in reaction buffer (110 mM Bicine-NaOH pH 8.0, 22 mM $MgCl_2$, 0.4 mM RuBP and ~100 W-A units of carbonic anhydrase), equilibrated with nitrogen (N_2), and containing one of nine concentrations of $NaH^{14}CO_3$ from 0 to 93 μM for the assays made from 10 to 25ºC and from 0 to 130 μM for the assays made at 30 and 40ºC, each with a specific radioactivity of 3.7 × 10^{10} Bq mol^{-1}, as described previously (Galmés et al. 2014). Assays (1.0 mL total volume) were started by injection of activated leaf extract and stopped after 60 s with the addition of 1 M formic acid. The acidified mixtures were dried and the ^{14}C products determined via scintillation counting, as described above. Rubisco K_c was determined from the fitted data as described elsewhere (Bird et al. 1982). Concentrations of CO_2 in solution in equilibrium with HCO_3^- were calculated assuming a pK_a for carbonic acid of 6.36, 6.25, 6.23, 6.21 and 6.19 at 10ºC, 20ºC, 25ºC, 30ºC and 40ºC, respectively, and using accurate measures of the pH of each buffer solution.

Temperature response

The temperature response of the Rubisco kinetic parameters from each species was modelled using a best fit of the data to the equation:

$$Parameter = Parameter_{(25^oC)} \exp\left[(T-25)E_a / 298\,R(T+273)\right]$$

where R is the molar gas constant (8.314 J K^{-1} mol^{-1}), E_a is the energy of activation, and T is the absolute assay temperature (von Caemmerer 2000).

Statistical analysis

Regressions between Rubisco kinetic parameters were calculated in Sigma Plot (v11). Photosynthetic mechanism differences in kinetic parameters and their activation energies were identified by ANOVA. Post hoc comparison among species was performed with Duncan test ($P < 0.05$) using Statistica 6.0 software package (StatSoft, Inc. USA).

Results

Variability of Rubisco kinetic parameters at 25ºC

At 25ºC, the carboxylase catalytic turnover rate of Rubisco (k_{cat}^c) varied between 2.48 s^{-1} in the C_3 *F. pringlei* and 3.42 s^{-1} in the C_4 *F. bidentis* (Table 1). The Rubisco Michaelis-Menten constant for CO_2 (K_c) ranged between 9.7 µM in the C_3 *F. pringlei* and 19.5 µM in the C_4 *F. bidentis* (Table 1). The differences in k_{cat}^c and K_c were compensatory, so that no interspecific differences were observed at 25ºC in the carboxylase catalytic efficiency, k_{cat}^c/K_c (data not shown). The Rubisco CO_2/O_2 specificity factor ($S_{c/o}$) was lowest in the C_4 *F. trinervia* (78.3 mol mol^{-1}) and highest in the C_3 *F. angustifolia* (90.4 mol mol^{-1}) (Table 1). The Michaelis-Menten constant for RuBP (K_{RuBP}) did not vary between these species (Table 1).

When the comparisons were made on the basis of the photosynthetic pathway, Rubisco from C_3 *Flaverias* had lower k_{cat}^c and K_c, and higher $S_{c/o}$ than Rubisco from C_4 species at 25ºC (Table 1). K_{RuBP} did not vary between photosynthetic pathways (Table 1).

Table 1. Parameter values (at 25ºC) and activation energies for the Rubisco carboxylase turnover rate (k_{cat}^c), the Michaelis-Menten constant for CO_2 (K_c), the specificity factor ($S_{c/o}$) and the Michaelis-Menten constant for RuBP (K_{RuBP}), for the six *Flaveria* species (n = 4-5 individual plants), and the average values for the three photosynthetic mechanisms (n = 8-10). Parameter estimates are derived from the regression of the Arrhenius equation (see Materials and Methods). Different letters denote statistically significant differences by Duncan's analysis ($P < 0.05$) among the photosynthetic mechanisms

Species	k_{cat}^c (s^{-1})		K_c (μM)		$S_{c/o}$ (mol mol^{-1})		K_{RuBP} (μM)	
	25ºC	E_a (kJ mol^{-1})	25ºC	E_a (kJ mol^{-1})	25ºC	E_a (kJ mol^{-1})	25ºC	E_a (kJ mol^{-1})
F. pringlei	2.48±0.27	53.9±1.8	11.4±1.0	41.6±2.6	90.1±1.4	-20.1±1.1	23.6±1.7	63.3±3.4
F. cronquistii	2.95±0.10	54.1±3.3	9.7±0.1	56.7±1.4	88.2±1.2	-22.2±0.7	22.3±1.1	67.1±3.3
Average C_3	2.71±0.16^A	54.0±1.8^B	10.6±0.6^A	49.1±3.6^B	89.1±0.9^B	-21.2±0.7^A	23.0±0.9^A	65.2±2.3^A
F. angustifolia	2.77±0.05	50.8±1.6	12.5±1.1	48.2±3.6	90.4±2.5	-25.6±2.0	20.2 ±0.5	65.7±1.4
F. floridana	2.86±0.10	52.5±1.4	11.9±0.2	43.1±0.6	84.3±1.4	-20.7±1.3	21.9±0.9	68.9±3.6
Average C_3/C_4	2.82±0.06^A	51.7±1.1^{AB}	12.5±0.6^B	46.0±2.1^B	87.3±1.8^B	-23.1±1.4^A	21.1±0.6^A	67.3±1.9^A
F. bidentis	3.42±0.14	47.6±1.6	19.5±1.2	35.6±7.3	80.0±0.3	-20.3±0.5	21.5±0.8	65.8±2.3
F. trinervia	3.28±0.20	48.8±2.8	18.4±0.8	35.6±3.0	78.3±1.6	-20.7±1.1	20.8±0.5	69.3±1.9
Average C_4	3.35±0.12^B	48.2±1.5^A	18.9±0.7^C	35.6±3.5^A	79.2±0.8^A	-20.5±0.6^A	21.1±0.5^A	67.6±1.6^A

The temperature response of Rubisco kinetic parameters

In all species, K_c, K_{RuBP}, and k_{cat}^c increased with assay temperature, while $S_{c/o}$ decreased at higher temperature (Fig. 1). These general trends were observed in all species, and therefore the differences in Rubisco kinetic parameters described at 25ºC were largely maintained between 10ºC and 40ºC (Fig. 1). For example, C_4 Rubiscos showed the highest k_{cat}^c and K_c at all assay temperatures, and no pathway differences were observed in K_{RuBP} at any assay temperature (Fig. 1). Differences in $S_{c/o}$ at 25ºC among Rubiscos from different photosynthetic pathways disappeared at 40ºC (Fig. 1c).

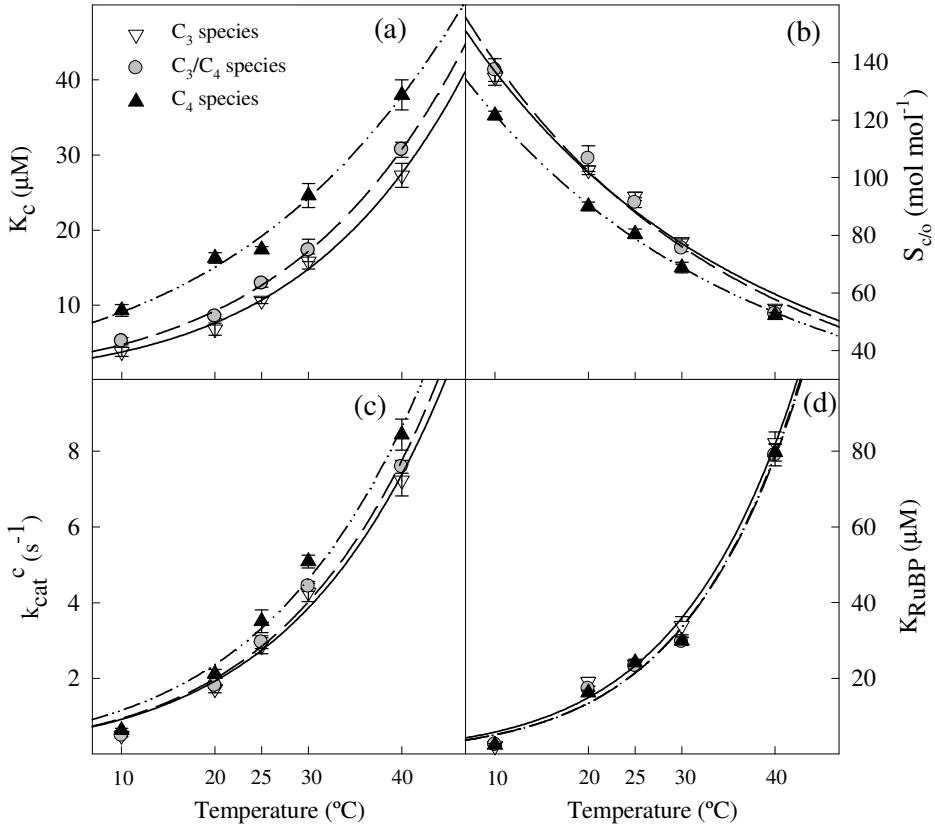

Figure. 1 The *in vitro* temperature response of the kinetic parameters of Rubisco from C_3 (downward empty triangles), C_3/C_4 intermediate (grey circles) and C_4 (upward black triangles) species of the genus *Flaveria*. (a) the Michaelis-Menten constant for CO_2, K_c; (b) the CO_2/O_2 specificity factor, $S_{c/o}$; (c) the carboxylase turnover rate, k_{cat}^c; and (d) the Michaelis-Menten constant for RuBP, K_{RuBP}. Lines represent the temperature response of the Rubisco kinetics parameters modelled using the equation described by von Caemmerer (2000, p. 45; See Materials and Methods), where solid, dashed and dashed-dotted lines corresponds to C_3, C_3/C_4 and C_4 species, respectively. Values are means ± standard errors (n = 4-5), with $P < 0.001$ for all regressions

To discern whether Rubiscos from species with contrasting photosynthetic mechanism present different thermal dependencies, we calculated the Arrhenius activation energies (E_a) of the Rubisco kinetic parameters. Significant within-pathway species differences in E_a were only observed for K_c between the C_3 species (Table 1). Among the photosynthetic mechanisms, C_4 Rubiscos had lower E_a for K_c than C_3 and photosynthetically intermediate Rubiscos (Table 1). The C_4 E_a of k_{cat}^c was nearly 11% lower than that of the C_3 enzymes, but was indistinguishable from that of the C_3-C_4 plants. The E_a of $S_{c/o}$ and K_{RuBP} did not differ between photosynthetic pathways (Table 1).

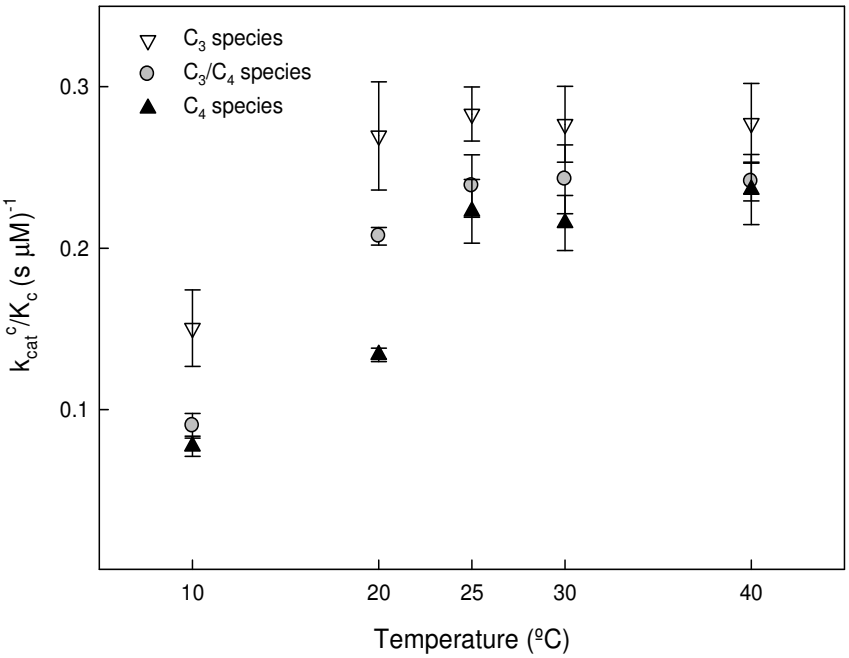

Fig. 2 The temperature response of the Rubisco carboxylation catalytic efficiency (k_{cat}^c/K_c) from C_3 (downward empty triangles), C_3/C_4 intermediate (grey circles) and C_4 (upward black triangles) species of the genus *Flaveria*. Values are means ± standard errors (n= 8-10)

Because the activation energy of k_{cat}^c was higher than that of K_c (Table 1), the carboxylation catalytic efficiency (k_{cat}^c/K_c) tended to increase with temperature, at least up to 25ºC (Fig. 2). In C_3 Rubiscos, for example, k_{cat}^c increased by 83% from 10ºC to 25°C, while K_c increased by only 64%, resulting in a 46% increase in the carboxylase catalytic efficiency (Fig. 2). Both k_{cat}^c and K_c increased by 60% from 25ºC to 40°C, constraining k_{cat}^c/K_c such that C_3 Rubisco is operating at maximum catalytic efficiency at 25°C. However, in C_4 Rubiscos, which have a lower E_a for both k_{cat}^c and K_c, the

relative increases in k_{cat}^c are always higher (Table 1). From 10ºC to 25°C, k_{cat}^c increased by 82%, while K_c increased only by 46%, resulting in a 64% increase in the carboxylase catalytic efficiency in C_4 Rubiscos. From 25ºC to 40°C, k_{cat}^c increased by 58%, while K_c increased only by 54%, resulting in an 8% increase in k_{cat}^c/K_c (Fig. 2).

The carboxylase substrate ratio (i.e., K_{RuBP}/K_c) tended to increase with temperature in all species. At 10ºC K_{RuBP}/K_c did not differ between Rubiscos, with values of 0.6 ± 0.2, 0.5 ± 0.1 and 0.3 ± 0.1 observed for C_3, C_3/C_4 and C_4 Rubiscos, respectively. By contrast, at 40ºC the differences among the photosynthetic mechanism were statistically significant, with K_{RuBP}/K_c values of 3.1 ± 0.2, 2.6 ± 0.2 and 2.1 ± 0.2 for C_3, C_3/C_4 and C_4, respectively.

We observed a negative curvilinear relationship between k_{cat}^c and $S_{c/o}$ ($r^2 = 0.98$, $P < 0.001$, Fig. S1a), and a positive linear relationship between k_{cat}^c and K_c ($r^2 = 0.92$, $P < 0.001$, Fig. S1b). The relationship between k_{cat}^c/K_c and $S_{c/o}$ was linear and negative ($r^2 = 0.44$, $P < 0.001$, data not shown), suggesting that the Rubisco oxygenase catalytic efficiency (k_{cat}^o/K_o) is much more sensitive to changes in temperature than k_{cat}^c/K_c.

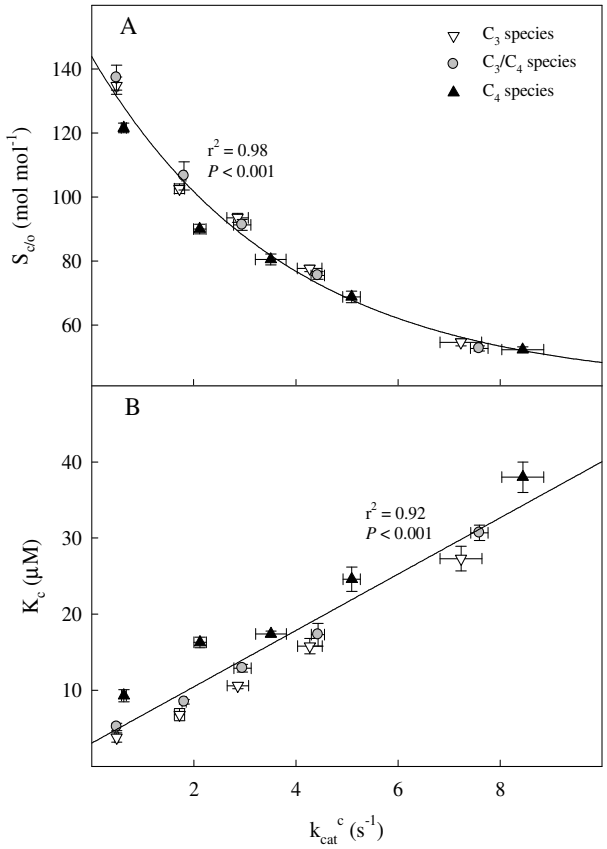

Figure S1. The relationship between the Rubisco carboxylase turnover rate (k_{cat}^c) and (A) the CO_2/O_2 specificity factor ($S_{c/o}$) and (B) the Michaelis-Menten constant for CO_2 (K_c) of C_3 (downward empty triangles), C_3/C_4 intermediate (grey circles) and C_4 (upward black triangles) species of the genus *Flaveria*.

The plots include data measured at five temperatures (10ºC, 20ºC, 25ºC, 30ºC and 40ºC). Values are means ± standard errors (n= 4-5).

Discussion

The results of the present study are consistent with previous data at 25ºC on the Rubisco biochemistry in *Flaveria* species (Kubien et al. 2008). Moreover, our data is in agreement with reports showing that Rubiscos from C_4 plants have higher k_{cat}^c than Rubiscos from C_3 species, and that this higher turnover comes at the expense of lower affinity for CO_2 (i.e., higher K_c) and lower $S_{c/o}$ (Wessinger et al. 1989; Sage 2002). Further, this study also confirms increases in k_{cat}^c and K_c, and a decrease in $S_{c/o}$, with increasing measurement temperature (Fig. 1) (Jordan and Ogren 1984; Brooks and Farquhar 1985; Uemura et al. 1997; Galmés et al. 2005). To our knowledge, the exponential increase of K_{RuBP} with increasing temperature (Fig. 1) has only been reported by Badger and Collatz (1977). However, we observed no differences in K_{RuBP} or its E_a. Similarly, Yeoh et al. (1981) found no K_{RuBP} differences between C_3 and C_4 plants. The lack of variation in K_{RuBP} suggests that steady-state levels of RuBP have not changed during the evolution of the C_4 photosynthetic pathway. By contrast, the ratio K_{RuBP}/K_c tends to increase with temperature as a consequence of the higher increase of K_{RuBP} relative to K_c (compare Fig. 1a and d). At high temperature, the reduction in the photosynthetic electron transport rate decreases the capacity to regenerate RuBP (Sage et al. 2008), and decreases in the RuBP pool size have been reported *in vitro* (Cen and Sage 2005). This fact, together with the observed increase in K_{RuBP}/K_c emphasizes RuBP regeneration as the prevalent photosynthetic limitation at high temperature.

The lower E_a for k_{cat}^c in Rubisco from C_4 versus C_3 species (Table 1) is in agreement with a previous report comparing two grass species (Kubien and Sage 2004). According to our results, K_c of Rubiscos from C_4 species is also less responsive to changes in temperature than Rubisco from C_3 species, with the consequence that there is no difference in the thermal sensitivity of $S_{c/o}$ between C_3 and C_4 Rubiscos (Table 1).

Differences in the temperature response of the carboxylation catalytic efficiency (k_{cat}^c/K_c) between C_3 and C_4 Rubiscos indicate that the C_4 version may be more able to respond to increases in temperature. This result is consistent with the higher optimum temperature for photosynthesis in C_4 than C_3 species (Yamori et al. 2014). Future surveys on the temperature dependency of other C_4 key enzymes like phosphoenolpyruvate carboxylase (PEPCase) would help deciphering the underlying

biochemical determinants for the enhanced performance of C_4 photosynthesis at high temperatures. By contrast, below 25ºC Rubiscos from C_3 species were more efficient carboxylases (i.e., higher k_{cat}^c/K_c) than Rubiscos from C_4 species. This observation is consistent with the higher photosynthetic efficiency and ecological dominance of C_3 species, with respect to C_4 species, in cool and temperate environments (Edwards et al. 2010). Because all the *Flaveria* species selected for this study are from tropical or sub-tropical habitats, this apparent adaptation of C_3 Rubiscos to low temperatures should be ascribed to the photosynthetic mechanism, and not to contrasting climatic origins of C_3 and C_4 species. The existence specific trends in response of Rubisco to changes in temperature should be considered to improve the accuracy of models aimed to predict the impact of temperature changes on the photosynthetic CO_2 assimilation.

The analysis of the molecular evolution of Rubisco in the *Flaveria* species reveals the existence of polymorphic residues under positive selection in the *rbc*L and *rbc*S genes encoding for the large (LSu) and small subunits (SSu) (Kapralov et al. 2011). In the LSu, the unique positively selected amino acid substitutions were at positions 149 and 309. Among the six *Flaveria* species included in the present study, the C_4 species *F. bidentis* and *F. trinervia* have ^{149}Ala/^{309}Ile , whereas the C_3 and C_3/C_4 species have ^{149}Asp/^{309}Met and ^{149}Glu/^{309}Met sequences, respectively (Kapralov et al. 2011). Therefore, the present results confirm previous data on the role of Met-309-Ile substitution as the C_4 catalytic switch in *Flaveria* Rubisco (Kapralov et al. 2011; Whitney et al. 2011), and suggests that this substitution may be also responsible for the contrasting temperature dependence of Rubisco kinetics between C_3 and C_4 *Flaveria* species. The role of substitution Asp/Glu-149-Ala is less clear. Whitney et al. (2011) showed that substitution Met-309-Ile alone was enough to interchange between C_3- and C_4-like kinetics, but changes at position 149 had no apparent effect on catalysis but rather seemed to influence holoenzyme biogenesis. This may explain the lower concentration of Rubisco in C_4 (^{149}Ala) as compared to C_3 (^{149}Glu, ^{149}Asp) *Flaveria* leaves. Future investigations through artificially designed tobacco transgenic lines expressing different *Flaveria* LSu would allow confirming the present results and exploring the putative role exerted by specific residue substitutions in SSu.

Author contributions

JAP, JG and DSK designed the research; JAP performed the in vitro measurements and the data treatment; APC supported the laboratory work. All authors analyzed the data and wrote the paper.

Acknowledgements

This work was partly supported by contract AGL2009-07999 (Plan Nacional, Spain) and a NSERC Discovery Grant (Canada, 327103-2008) awarded to JG and DSK, respectively. JAP was recipient of a FPI grant from the Govern de les Illes Balears. We would like to acknowledge Dr. Maxim Kapralov (Australian National University) for his helpful comments on a previous version of the manuscript, Dr. Spencer Whitney (Australian National University) for his comment on residue 149 and Dr. Josep Cifre (Universitat de les Illes Balears) for his statistical advice.

References

Ainsworth EA, Ort DR (2010) How do we improve crop production in a warming world? Plant Physiol 154:526–30

Andrews T, Lorimer G (1987) Rubisco: structure, mechanisms, and prospects for improvement. In: Hatch M, Boardmaneds N (eds) The Biochemistry of Plants: A Comprehensive Treatise, Vol 10, Photosynthesis. Academic Press, New York, pp. 131–218

Badger M, Collatz G (1977) Mechanism of ribulose-1,5-bisphosphate carboxylase and oxygenase reactions, with particular reference to the effect of temperature on kinetic parameters. Carnegie Inst Washington, Year B 76:355–361

Berry J, Björkman O (1980) Photosynthetic response and adaptation to temperature in higher-plants. Physiol Plant Mol Biol 31:491–543

Bird I, Cornelius M, Keys A (1982) Affinity of RuBP Carboxylases for Carbon Dioxide and Inhibition of the Enzymes by Oxygen. J Exp Bot 33:1004–1013

Brooks A, Farquhar GD (1985) Effect of temperature on the CO_2/O_2 specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase and the rate of respiration in the light. Planta 165:397–406

Bunce JA (2000) Acclimation of photosynthesis to temperature in eight cool and warm climate herbaceous C_3 species: Temperature dependence of parameters of a biochemical photosynthesis model. Photosynth Res 63:59–67

Cen Y, Sage R (2005) The regulation of Rubisco activity in response to variation in temperature and atmospheric CO_2 partial pressure in sweet potato. Plant Physiol 139:979–990

Christin PA, Salamin N, Muasya AM, Roalson EH, Russier F, Besnard G (2008) Evolutionary switch and genetic convergence on *rbc*L following the evolution of C_4 photosynthesis. Mol Biol Evol 25:2361–2368

Edwards EJ, Osborne CP, Strömberg CAE, Smith SA, Bond WJ, Christin PA, et al. (2010) The origins of C_4 grasslands: integrating evolutionary and ecosystem science. Science 328:587–591

Flexas J, Ribas-Carbó M, Diaz-Espejo A, Galmés J, Medrano H (2008) Mesophyll conductance to CO_2: current knowledge and future prospects. Plant Cell Environ 31:602–21

Galmés J, Flexas J, Keys AJ, Cifre J, Mitchell R, Madgwick PJ, Haslam RP, Medrano H, Parry MAJ (2005) Rubisco specificity factor tends to be larger in plant species from drier habitats and in species with persistent leaves. Plant Cell Environ 28:571–579

Galmés J, Kapralov M V, Andralojc PJ, Conesa MÀ, Keys AJ, Parry MAJ, Flexas J (2014) Expanding knowledge of the Rubisco kinetics variability in plant species: environmental and evolutionary trends. Plant Cell Environ 1–46

Galmés J, Perdomo JA, Flexas J, Whitney SM (2013) Photosynthetic characterization of Rubisco transplantomic lines reveals alterations on photochemistry and mesophyll conductance. Photosynth Res 115:153–166

Hall NP, Keys AJ (1983) Temperature dependence of the enzymic carboxylation and oxygenation of ribulose 1,5-bisphosphate in relation to effects of temperature on photosynthesis. Plant Physiol 72:945–948

Hikosaka K, Ishikawa K, Borjigidai A, Muller O, Onoda Y (2006) Temperature acclimation of photosynthesis: mechanisms involved in the changes in temperature dependence of photosynthetic rate. J Exp Bot 57:291–302

IPCC (2013) Working Group I Contribution to the IPCC Fifth Assessment Report Climate Change 2013: The Physical Science Basis Summary for Policymakers. 1–36

Jordan DB, Ogren WL (1984) The CO_2/O_2 specificity of ribulose 1,5-bisphosphate carboxylase/oxygenase. Planta 161:308–313

Kane H, Viil J, Entsch B, Paul K, Matthew K, Andrews T (1994) An improved method for measuring the CO_2/O_2 specificity of ribulosebisphosphate carboxylase-oxygenase. Aust J Plant Physiol 21:449–461

Kane H, Wilkin J, Portis A, Andrews T (1998) Potent inhibition of ribulose-bisphosphate carboxylase by an oxidized impurity in ribulose-1,5-bisphosphate. Plant Physiol 117:1059–1069

Kapralov MV, Kubien DS, Andersson I, Filatov DA (2011) Changes in Rubisco kinetics during the evolution of C_4 photosynthesis in *Flaveria* (Asteraceae) are associated with positive selection on genes encoding the enzyme. Mol Biol Evol 28:1491–1503

Kapralov MV, Smith JAC, Filatov DA (2012) Rubisco evolution in C_4 eudicots: an analysis of Amaranthaceae *Sensu Lato*. PLoS One 7:e52974

Kubien DS, Brown C, Kane H (2011) Quantifying the amount and activity of Rubisco in leaves. In: Carpentiered R (ed) Methods in molecular biology 684: Photosynthesis research protocols. Humana Press, New York, pp. 349–362

Kubien DS, von Caemmerer S, Furbank R, Sage R (2003) C_4 photosynthesis at low temperature. A study using transgenic plants with reduced amounts of Rubisco. Plant Physiol 132:1577–1585

Kubien DS, Sage R (2004) Low-temperature photosynthetic performance of a C_4 grass and a co-occurring C_3 grass native to high latitudes. Plant Cell Environ 27:907–916

Kubien DS, Whitney SM, Moore PV, Jesson LK (2008) The biochemistry of Rubisco in *Flaveria*. J Exp Bot 59:1767–1777

Paul K, Morell M, Andrews T (1991) Mutations in the small subunit of ribulose bisphosphate carboxylase affect subunit binding and catalysis. Biochemistry 30:10019–10026

Ruuska S, Andrews T, Badger M, Hudson G, Laisk A, Price GD, von Caemmerer S (1998) The interplay between limiting processes in C_3 photosynthesis studied by rapid-response gas exchange using transgenic tobacco impaired in photosynthesis. Aust J Plant Physiol 25:859–870

Sage RF (2002) Variation in the k_{cat} of Rubisco in C_3 and C_4 plants and some implications for photosynthetic performance at high and low temperature. J Exp Bot 53:609–620

Sage RF, Kubien DS (2007) The temperature response of C_3 and C_4 photosynthesis. Plant Cell Environ 30:1086–1106

Sage RF, Way DA, Kubien DS (2008) Rubisco, Rubisco activase, and global climate change. J Exp Bot 59:1581–1595

Sander R (1999) Modeling atmospheric chemistry: Interactions between gas-phase species and liquid cloud/aerosol particles. Surv Geophys 20:1–31

Sharkey TD (2005) Effects of moderate heat stress on photosynthesis: importance of thylakoid reactions, Rubisco deactivation, reactive oxygen species, and thermotolerance provided by isoprene. Plant Cell Environ 28:269–277

Shay PE, Kubien DS (2013) Field analysis of photoprotection in co-occurring cool climate C_3 and C_4 grasses. Physiol Plant 147:316–328

Tieszen L, Sigurdson D (1973) Effect of temperature on carboxylase activity and stability in some Calvin cycle grasses from the arctic. Arct Alp Res 5:59–66

Uemura K, Anwaruzzaman, Miyachi S, Yokota A (1997) Ribulose-1,5-bisphosphate carboxylase/oxygenase from thermophilic red algae with a strong specificity for CO_2 fixation. Biochem Biophys Res Commun 233:568–71

von Caemmerer S (2000) Biochemical models of leaf photosynthesis. CSIRO, Collingwood.

Weber D, Andersen W, Hess S, Hansen D, Gunasekaran M (1977) Ribulose-1,5-bisphosphate carboxylase from plants adapted to extreme environments. Plant Cell Physiol 18:693–699

Wessinger M, Edwards G, Ku M (1989) Quantity and kinetic properties of ribulose-1,5-bisphosphate carboxylase in C_3, C_4, and C_3-C_4 intermediate species of *Flaveria* (Asteraceae). Plant cell Physiol 30:665–671

Whitney SM, Sharwood RE, Orr D, White SJ, Alonso H, Galmés J (2011) Isoleucine 309 acts as a C_4 catalytic switch that increases ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylation rate in *Flaveria*. Proc Natl Acad Sci USA 108:14688–14693

Yamori W, Hikosaka K, Way DA (2014) Temperature response of photosynthesis in C_3, C_4, and CAM plants: temperature acclimation and temperature adaptation. Photosynth Res 119:101–117

Yamori W, Suzuki K, Noguchi K, Nakai M, Terashima I (2006) Effects of Rubisco kinetics and Rubisco activation state on the temperature dependence of the

photosynthetic rate in spinach leaves from contrasting growth temperatures. Plant Cell Environ 29:1659–1670

Yeoh HH, Badger MR, Watson L (1981) Variations in Kinetic Properties of Ribulose-1,5-bisphosphate Carboxylases among Plants. Plant Physiol 67:1151–5

Zhu G, Jensen RG, Bohnert HJ, Wildner GF, Schlitter J (1998) Dependence of catalysis and CO_2/O_2 specificity of Rubisco on the carboxy-terminus of the large subunit at different temperatures. Photosynth Res 140:71–79

Chapter 7

GENERAL DISCUSSION

This PhD Thesis studied the effects of two main variables of global climate change –temperature and water availability– on growth, physiology and biochemistry of three crop plants and six *Flaveria* species with contrasting photosynthetic mechanisms. The results obtained in this research, along with the corresponding specific discussion, have been structured in four chapters (3, 4, 5 and 6). The present chapter contains a general discussion from the most important results presented in the chapters mentioned above, to advance on the major conclusions of this work.

7.1. Effects of water deficit and high temperature: individual vs. combined stress on growth, physiological and biochemical processes

Climate change predict an increase in global temperature, changing the distribution of precipitation and intensifying drought in arid and semiarid areas (Chaves, Maroco & Pereira 2003; IPCC 2013). As pointed out in the Introduction, water deficit and high temperature are important environmental factors restricting plant growth and photosynthesis in many regions of the world, and the two stresses often occur simultaneously, particularly in Mediterranean agricultural areas (Shah & Paulsen 2003; Rizhsky *et al.* 2004; Bates *et al.* 2008; Habash, Kehel & Nachit 2009).

The results obtained in the present Thesis largely confirm the detrimental effect of high temperature (HT) and water deficit (WD) on the leaf physiology and plant growth on three of the more important crops worldwide, rice, wheat and maize. So far, few investigations have studied the interactions between the two stresses (Rizhsky *et al.* 2004; Prasad *et al.* 2011; Vile *et al.* 2012); instead, the effects of WD and HT have been typically analyzed separately (Porter & Gawith 1999; Wheeler *et al.* 2000; Prasad *et al.* 2006; Prasad, Staggenborg & Ristic 2008).

Table 1 summarizes the effects of WD, HT and its interaction on the main growth, physiological and biochemical parameters measured in this study (parameters from chapters 3, 4 and 5). The effects of WD and HT on the parameters studied differ in magnitude depending on the species. Compared with the control conditions – plants growth at 25ºC and well-watered – total plant biomass (B_t) was large affected by HT and WD in all three crops, with a higher impact of the combined stress. This result is in accordance with recent findings (Prasad *et al.* 2011; Vile *et al.* 2012) and highlights the importance of considering the interactive effects of these two stresses to better predict the negative impacts on plant's performance. The changes in plant growth under both

stresses were evaluated with regard its morphological components (Table 1). WD and HT presented contrasting effects on patterns of morphological components in a species dependent–manner, being maize the species with fewer changes in its morphology under both stresses and its combination. On morphological parameters the effects of HT and WD were additives in rice and maize due to the lack of significant interaction between both stresses, being these mostly influenced by HT. This finding is in line with the results from Vile *et al.* (2012) in Arabidopsis that showed a general independency between the mechanisms involved in the responses to these stresses. By contrast, wheat showed an interactive effect of HT and WD on its morphological parameters (Table 1).

In this study, WD exerted a detrimental effect in most of the physiological parameters in the three crops, but especially in rice and maize. This fact shows that under WD the decrease in carbon gain is principally due to an increase in diffusive and metabolic limitations, with a greater detrimental effect on diffusive parameters. These patterns have been also observed by other authors in other species (Chaves *et al.* 2003; Flexas *et al.* 2004, 2008; Grassi & Magnani 2005; Galmés, Medrano & Flexas 2007). At HT the net CO_2 assimilation (A_N) decreased in both C_3 species. However, HT was less detrimental regarding to the physiological parameters in rice and maize, though wheat was most affected by HT. Metabolic parameters as V_{cmax} and J_{max} increased in both C_3 species, which is in line with other reports (Yamori, Noguchi & Terashima 2005; Hikosaka *et al.* 2006; Carmo-Silva *et al.* 2012; Joseph, Whitehead & Turnbull 2014). Therefore, decreases in A_N at HT could be related to an increase in photorespiration (Kubien & Sage 2008). Although some physiological parameters were affected only by one or the other stress, being this species–specific. The interactive effects of HT and WD in the three species was significant in most of the physiological parameters. Therefore, this would have important consequences for modelling plant growth under combined stresses.

There is much variability on Rubisco parameters reported on literature, which has been attributed to differences in the velocity of stress imposition and to species specific responses (Parry *et al.* 2002; Flexas *et al.* 2006b; Galmés *et al.* 2011b). Galmés *et al.* (2013) has reviewed not significant or only minor changes in any of the Rubisco-related parameters under mild-to-moderate drought, which is consistent with the results observed in this study on Rubisco amount and carbamylation in the three species (Table 1). However, the combined effect of WD and HT was not more detrimental, which shows independence in the effect of each stress and a higher impairment by HT than by

WD. Decreases in Rubisco carbamylation are highly linked with Rubisco activase (Rca); since it presents an optimum temperature lower than Rubisco (Salvucci *et al.* 2001). However, the amount of the large and small isoforms of Rca remained unchanged or even increased under WD and HT in the three species, with only an exception in wheat at HT that showed a slight decrease on the large Rca isoform (Table 1). Therefore, the amount of Rca could not be responsible for the decrease of Rubisco carbamylation. However, we cannot rule out the possibility that the activation of Rca would be inhibited by the increase in temperature, therefore, the inactivation of Rca appears to be a direct effect of temperature on the thermal stability of the protein (Barta *et al*. 2010, Carmo-silva & Salvucci 2012).

Finally, the leaf carbon balance (LCB) was calculated as the integration of the daily CO_2 assimilation rate over 24 h (see Chapter 3). LCB gives an indication of the efficiency of plant carbon use (Loveys *et al*. 2002), and it is also indicative of the capacity of plants to produce new biomass (Cavaleri *et al.* 2008). WD and HT showed a detrimental effect on LCB in the three crops and a larger impaired effect by the combination of both stresses (Table 1). This is due to an enhancement of the rate of respiratory CO_2 release, coupled with inhibited CO_2 assimilation. This decreases in LCB were highly related with decreases in biomass. Therefore, The net CO_2 assimilation (A_N) and the mitochondrial respiration (R) are the most fundamental plant physiological processes governing carbon balance and biomass production (Atkin, Scheurwater & Pons 2007; Cavaleri, Oberbauer & Ryan 2008).

Table 1. Summary of the effects of high temperature (HT), water deficit (WD) and its interaction (HT×WD) on the main parameters considered in the present Thesis, in plants of rice, wheat and maize. The net CO_2 assimilation (A_N), the stomatal conductance (g_s), the intrinsic water use efficiency (A_N/g_s) and the mitochondrial respiration (R) were measured at mid–morning under light–saturating conditions, as is explained in chapter 3.

Parameters		Rice			Wheat			Maize		
		HT	WD	HT×WD	HT	WD	HT×WD	HT	WD	HT×WD
Growth parameters	B_t	↓	↓	↓↓	↓↓	↓↓	↓↓↓	↓↓	↓↓	↓↓
	LMR	↑↑	=	↑↑	↑↑	↑↑	↑↑↑	=	↑	=
	LMA	↓↓	=	↓↓	↓	↑	↓	=	=	=
	LAR	↑↑↑	=	↑↑↑	↑↑↑	↑↑	↑↑↑	=	↑	=
Physiological parameters	RWC	=	=	=	=	=	=	=	=	↓
	F_v/F_m	=	=	=	↓	=	=	=	=	=
	iA_N	↓	↓↓	↓↓	↓	↓	↓↓	=	↓	↓
	ig_s	↑	↓↓↓	↓↓	↓	↓↓↓	↓↓	↑	↓	↓
	g_m	↓	↓↓	↓↓	↓↓	↑↑↑	↓	-	-	-
	iA_N/ig_s	↓	↑↑↑	↑	↓	↑↑↑	↑↑	=	↑	↑
	iR	↑↑↑	↑	↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑	↑↑
	LCB	↓↓	↓↓	↓↓↓	↓↓	↓↓↓	↓↓↓	↓	↓↓	↓↓↓
	C_i	=	↓	↓	=	↓↓	↓	=	↓	↓
	C_c or C_s	=	↓	↓	↑	↓	=	↑	↓↓	↓↓
	V_{cmax}	↑↑↑	↓	↑↑	↑↑↑	↑	↑↑↑	=	↓	=
	J_{max} or V_{pmax}	↑	↓	=	=	=	=	↑↑	↓	↑
Biochemical parameters	[Rubisco]	↓	↓	↓↓	↓	↓	↓	↓	↓↓	↓
	Carbamylation	↓↓↓	↓	↓↓↓	↓↓	=	↓↓	↓↓	↑↑↑	↓↓
	[Rca large]	↑	=	=	↓	↑	↑↑	-	-	-
	[Rca small]	=	↑	↑	↑↑	↑↑	↑↑↑	=	=	↑

= Means 90-110% of the value measured under the control treatment (WW×CT), ↓ 60-90%, ↓↓ 30-60%, ↓↓↓ < 30%, ↑ 110-140%, ↑↑ 140-170%, ↑↑↑ > 170%, and - data not evaluated.

Table 2 shows general trends of variation in the main group parameters under the different treatments. Both C_3 species showed a similar sensitivity to HT on growth parameter, with a 13% of variation with regard to the control conditions, while maize only presented a 4% of variation. Therefore, maize was the species less affected by the increase of temperature in terms of morphological parameters, because the reduction in B_t at HT is higher in maize than in rice. Under WD and HT×WD, wheat was the species more sensitive on growth parameters, with a 12% and 17% of variation regarding the control. This behaviour in wheat has been also seen in other studies (Shah & Paulsen 2003; Nagai & Makino 2009; Prasad *et al.* 2011). Regarding the physiological parameters, wheat was again the species with the largest percentage of variation under all treatments, where WD showed the highest variation (Table 1). Biochemical parameters were mostly sensible to the combination of both stresses in the two C_3 species (Table 2). These effects of both stresses on the three crops shows that some traits were specific and dependent on the species, such as it has been reported in literature (Yamori *et al.* 2009, 2010). The summation of the different groups of parameters shows that wheat is the species more sensitive to the different stresses, in particular to the combination of HT×WD. That performance in wheat might be attribute to the fact that its ancestors were originated in cold-temperate environments, which makes this more sensitive to HT (Nagai & Makino 2009). Therefore, this results indicate that the origin of the species is an important aspect in plant acclimation to new detrimental conditions (Yamori *et al.* 2010).

Table 2. Percentage (%) of variation of the growth, physiological and biochemical group of parameters under water deficit (WD), high temperature (HT) and their combination (HT×WD) with regard to the control conditions, in rice, wheat and maize. The percentage of each group of parameters was calculated dividing the sum of the effects of each parameter of the group (one arrow=1, two arrows=2 and three arrows=3) obtained in the Table 1, by the potential maximum number of arrows under each stress (60 in the case of rice and wheat and 54 in maize).

Group of parameters	Rice			Wheat			Maize		
	HT	WD	HT×WD	HT	WD	HT×WD	HT	WD	HT×WD
Growth parameters	13	2	15	13	12	17	4	7	4
Physiological Parameters	22	28	25	25	33	27	14	21	22
Biochemical parameters	8	5	10	10	7	13	6	9	7
Total	**43**	**35**	**50**	**48**	**52**	**57**	**24**	**37**	**33**

7.2. Effects of high temperature on photosynthetic parameters: short– *vs*. long–term exposition

Under natural conditions, the different stresses normally develop gradually, over periods comprising weeks or months, allowing plant acclimation responses to occur (Flexas *et al.* 2006a). Therefore, a goal of this research was to compare the commonly applied short–term treatments with plants subjected to long–term stressful conditions. The different responses between plants exposed to short– and long–term stress rely on the development of acclimation processes (Prasad *et al.* 2011; Vile *et al.* 2012).

The results of the long–term (LT) and short–term (ST) temperature exposition on physiological parameters (Chapter 4) are summarized in Table 3. The A_N at LT showed a better acclimation in maize than in both C_3 species, but at ST A_N was overestimated in maize and underestimated in wheat. These results demonstrate a better acclimation of maize to HT. This behavior has long been recognized, because the C_4 plants have a higher temperature optimum than C_3 (Yamori, Hikosaka & Way 2014),

and in C3 species A_N is usually inhibited when leaf temperatures exceed about 38°C (Berry & Björkman 1980; Edwards & Walker 1983; Crafts-brandner & Salvucci 2002). There are very few surveys in literature analyzing the interaction between the plant growth temperature and the measurement temperature of physiological parameters. Yamori *et al.* (2006) showed in spinach that g_s and g_m responded similarly, where both parameters increase as measurement temperature rises in plants grown at 15ºC and 30ºC. Spinach, as wheat, was originated in temperate environments (Salvucci & Crafts-brandner 2004) and in the data obtained in this study both g_s and g_m increased in wheat plants grown and measured at HT with regard to plants grown and measured at CT (for details see Chapter 4). However, at ST g_s remained unchanged, which indicate that g_s may be overestimated at ST in wheat (Table 3). Despite of the differences observed in both conductances under LT HT, the C_i reached a perfect homeostatic adjustment at LT and ST. R shows a largest increase in the three species at LT and ST, which exhibit that the increase in R with temperature is independently of the time of exposition (Table 3).

The optimum temperature of photosynthesis is generally between 20 and 30ºC, while the optimum temperature of respiration occurs just below the temperature at which heat inactivation of enzymes occurs (e.g., above 45ºC). Therefore, some authors has reported that A_N shows a less acclimation potential to a change in temperature than R (Atkin & Tjoelker 2003; Yamori *et al.* 2005; Campbell *et al.* 2007; Ow *et al.* 2008; Way & Sage 2008). In both C_3 species, A_N decreased at LT and ST at time that R were increased under LT and ST, being wheat the species with the most negative balance between A_N and R at ST. These results determine the carbon balance at leaf level, and hence may give an indication about the growth capacity of these species at higher temperature (Flexas *et al.* 2006a; Atkin & Macherel 2009; Zhao *et al.* 2013). Thus, it is important to obtain a positive balance between respiration and photosynthesis, because these processes ranging from plant productivity to ecosystem balance and the flux of carbon through the biosphere.

Metabolic parameters as V_{cmax} increased largely in rice and wheat at LT (Table 3). This tendency is in agreement with Yamori *et al.* (2006) that found that plants grown and measured at same temperature performed more efficiently. However, wheat did not show the same increase in V_{cmax} at ST, which suggest that this species is more vulnerable to rapid changes in temperature and could be in part responsible for the decrease in A_N at ST. In maize, V_{cmax} remained unchanged at LT and only showed a small increase at ST (Table 3). This capacity of acclimation at long–term for the three

species is in agreement with literature reports that say that growth temperature caused changes in various physiological characteristics, but the extent of plasticity differed depending on plant species and physiological characteristics (Kubien & Sage 2004; Osborne *et al.* 2008; Yamori *et al.* 2010, 2014).

Table 3. Comparison of the effects of long– and short–term (LT and ST) high temperature (HT), on some parameters considered in the present Thesis, in plants of rice, wheat and maize. The LT values resulted dividing the plants grown at control temperature and measured at 25ºC (CT-25ºC) with the plants grown at high temperature measured at 38ºC (HT-38ºC). The ST was obtained dividing the plants grown at CT measured at 25ºC with the plants measured at 38ºC (CT-25ºC and CT-38ºC).

Parameter	Rice		Wheat		Maize	
	HT (LT)	HT (ST)	HT (LT)	HT (ST)	HT (LT)	HT (ST)
A_N	↓	↓	↓	↓↓	=	↑
g_s	↑	↓	↓	=	↑	↑↑
R	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑
C_i	=	=	=	↑	=	↑
g_m	↓	↓↓	↓↓	↓↓	-	-
C_c or C_s	=	=	↑	↑	↑	↓↓
V_{cmax}	↑↑↑	↑↑↑	↑↑↑	↑	=	↑
J_{max} or V_{pmax}	↑	↑	=	↓	↑↑	=

= Means 90-110% of the value measured under the control treatment (WW×CT), ↓ 60-90%, ↓↓ 30-60%, ↓↓↓ < 30%, ↑ 110-140%, ↑↑ 140-170%, ↑↑↑ > 170%, and - data not evaluated.

7.2.1. Rubisco acclimation to high temperature and water deficit.

The acclimation on the biochemical parameters of photosynthesis might be related with metabolic changes, including the modulation of the expression of photosynthesis–related genes (Chaves, Flexas & Pinheiro 2009; Pinheiro & Chaves 2011). For instance, it has been suggested that the acclimation on Rubisco biochemical

characteristics is mediated by variable gene expression (Cavanagh & Kubien 2014). The kinetic parameters describing Rubisco function vary among species (Jordan & William 1981; Yeoh, Badger & Watson 1981; Bird, Cornelius & Keys 1982; Jordan & Ogren 1984; Delgado *et al.* 1995; Kubien *et al.* 2008), which may reflect adaptation to local environments (Delgado *et al.* 1995; Sage 2002; Galmés *et al.* 2005, 2014; Savir *et al.* 2010). In addition, the expression of Rubisco–coding genes varies in response to the environment (Dedonder *et al*. 1993; Cheng *et al*. 1998; Yoon *et al*. 2001; Yamori *et al*. 2006), suggesting a selection for a rapid acclimation response.

In rice and wheat, SSu are encoded by a nuclear multigene family (*rbc*S) with some differences in the peptide sequence (Silverthorne & Tobin 1990). In particular, 5 and at least 10 different *rbc*S genes have been described in rice (Kikuchi *et al*. 2003) and wheat (Broglie *et al*. 1983), respectively. It has been described that expression of *rbc*S genes can be controlled by environmental factors, such as the irradiance (Tobin & Silverthorne 1985; Kuhlemeier *et al*. 1987; Spreitzer 2003) and growth temperature (Yoon *et al*. 2001). On this basis, and the demonstration that SSu influences Rubisco kinetics (Spreitzer 2003), future efforts should be focused to unravel the environmental modulation of *rbc*S expression and its effects on Rubisco functioning.

Attending to the data in Fig. 4 on Chapter 4 (A_G vs $C_{c/O}$), the potential role of SSu acquires even more importance. This figure shows the relationship between gross photosynthesis (A_G) and the ratio between the gaseous substrates of Rubisco (C_c/O). For simplicity, Fig. 4 represents only correlation lines for some treatments. In spite of this, it can be seen that wheat plants presented different A_G depending on the temperature of measurement, with measures at 25ºC presenting higher values as compared to those from plants measured at 38ºC. It must be remarked that A_G is governed by the rates of carboxylation (v_c) and oxygenation (v_o), which in turn are determined, at a given C_c/O, by the Rubisco specificity factor ($S_{c/o}$) (von Caemmerer 2000). Because $S_{c/o}$ decreases with temperature (Brooks & Farquhar 1985; Galmés *et al.* 2005), it is not surprising that A_G responds accordingly (Fig. 4). Moreover, at the same temperature of measurement, plants grown at different temperature displayed somewhat different A_G (Fig. 4), suggesting that $S_{c/o}$ may have been changed. This 'thermal acclimation' of Rubisco kinetics may be mediated by the differential composition of SSu, and has been demonstrated to occur in spinach acclimated at different temperatures (Yamori *et al.* 2006).

7.3. The role of Rubisco kinetics in photosynthesis modelling

Biochemical models as the described by Farquhar *et al.* (1980) for C_3 plants and by von Caemmerer (2000) for C_4 plants are used to predict the impact of climate change on rates of net CO_2 assimilation, scaling from individual leaves all the way up to canopies and ecosystems (Long 1991; De Pury & Farquhar 1997; Cramer *et al*. 2001). These models are driven by the impact of CO_2 availability and temperature on the activity of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco). However, these models still suffers from insufficient information on the full parameterization of the temperature response of photosynthesis (Leuning 2002; Medlyn *et al.* 2002a; Medlyn, Loustau & Delzon 2002b). In particular, the Rubisco kinetics and maximum activity that are important determinants of leaf CO_2 exchange and are fundamental components of leaf models of photosynthesis (Farquhar *et al.* 1980; von Caemmerer & Farquhar 1981).

7.3.1. The importance to use the species–specific Rubisco kinetics in the biochemical models of photosynthesis

Photosynthesis has been modelled in many different species, ranging from trees as *Olea europea* to herbaceous as *Arabidopsis thaliana* (see Table 4), using the tobacco Rubisco kinetics obtained by von Caemmerer *et al.* (1994) and Bernacchi *et al.* (2002). However, it is well documented that significant differences occur among species in Rubisco kinetics (e.g., Galmés *et al.* 2005; Savir *et al.* 2010; Galmés *et al.* 2014), and recent papers have pointed out the necessity of include this variability in models if we want to simulate and understand the photosynthetic performance of these species in their natural environments (Diaz-Espejo 2013; Walker *et al.* 2013).

Current models of leaf photosynthesis estimated the Rubisco specificity factor ($S_{c/o}$) indirectly from the CO_2 compensation point in the absence of mitochondrial respiration (Γ^*) after gas exchange measurements (Bernacchi *et al.* 2001). Actually, Γ^* is estimated following the method described by Laisk (1977), this estimations are strongly affected by lateral leakage through the IRGA's chamber gaskets as well as by cuticular conductance to CO_2 and water. This suggest that in plants with restricted photosynthesis – e.g. under WD or HT – it would be better to determine $S_{c/o}$ independently of gas–exchange measurements, using alternative methods (Warren,

Livingston & Turpin 2004; Galmés, Medrano & Flexas 2006). In turn, a controversy is open about if the Rubisco kinetic parameters should be estimate by *in vivo* or *in vitro* techniques, to be then applied to the biochemical models. For instance, Walker *et al*. (2013) argued that Rubisco kinetics constants for both CO_2 and O_2 were significantly over estimated *in vitro* with regard to the *in vivo* at 25ºC in *N. tabacum.* The determination of *in vivo* kinetics for a large number of species with different functional types is urgent, as advised by Walker *et al*. (2013), but this not be accomplished in the short term due to the complexity to apply the techniques to obtain the *in vivo* Rubisco kinetics. However, measuring *in vitro* kinetic constants of Rubisco is less complex methodologically, so that a number of different species can be characterised in a reasonable time. Hence, in this thesis the *in vitro* Rubisco kinetic constants were calculated ($S_{c/o}$, K_c and K_o) for the three species with the aim to be more accurate with the photosynthetic models. The kinetic values that we obtained in the three crops were within the range of values obtained *in vivo* for tobacco and Arabidopsis by Walker *et al.* (2013) demonstrating a non–overestimation in our data suggesting that using *in vitro* values is a valid approach to estimate Rubisco constants.

The Michaelis–Menten constant for PEPc (K_p) for maize was calculated to be applied to the C_4 photosynthetic model by von Caemmerer (2000). Also, the 'standard' Rubisco and PEPc kinetics reported by Bernacchi *et al.* (2002) in C_3 plants and by von Caemmerer (2000) in C_4 plants were used to compare the results obtained from the same model using different parameterization (see Chapter 4). Thus, the use of each species Rubisco constants resulted in model parameterization estimates that in many cases differ significantly from those obtained using the 'standard' constants by Bernacchi *et al.* (2002) in C_3 plants. The differences observed in this thesis were a generalised overestimation of V_{cmax} and a largely variable underestimation of J_{max} when was using the "standard" tobacco constants by Bernacchi *et al.* (2002) in both C_3 plants.

These discrepancies found in close related species such as rice and wheat in this Thesis and tobacco and Arabidopsis in Walker *et al.* (2013), ratified the importance of take into account the Rubisco kinetics parameter from each species in the photosynthetic models.

Table 4. A list with some examples from different authors using the "standardized" Rubisco kinetics reported by von Caemmerer *et al.* (1994) and Bernacchi *et al.* (2002) to model photosynthesis in different species.

Species studied	Reference	Original paper
Nicotiana tabacum	von Caemmerer *et al.* (1994)	von Caemmerer *et al.* (1994)
Nicotiana tabacum	Bernacchi *et al.* (2002)	Bernacchi *et al.* (2002)
Olea europea	Loreto *et al.* (2003)	Bernacchi *et al.* (2002)
Fagus sylvatica.	Dreyer *et al.* (2001)	von Caemmerer *et al.* (1994)
Betula pendula	Dreyer *et al.* (2001)	von Caemmerer *et al.* (1994)
Juglans regia	Dreyer *et al.* (2001)	von Caemmerer *et al.* (1994)
Quercus robur	Dreyer *et al.* (2001)	von Caemmerer *et al.* (1994)
Quercus petrea	Dreyer *et al.* (2001)	von Caemmerer *et al.* (1994)
Arabidopsis thaliana	Flexas *et al.* (2007)	Bernacchi *et al.* (2002)
Nicotiana sylvestris	Galle *et al.* (2009)	Bernacchi *et al.* (2002)
Solanum lycopersicum	Galmés *et al.* (2011)	Bernacchi *et al.* (2002)
Helianthus annuus	Archontoulis *et al.* (2012)	Bernacchi *et al.* (2002)
Hibiscus cannabinus	Archontoulis *et al.* (2012)	Bernacchi *et al.* (2002)
Cynara cardunculus	Archontoulis *et al.* (2012)	Bernacchi *et al.* (2002)

7.3.2. The importance to knowing the thermal dependence of Rubisco kinetic parameters for use on the photosynthesis models

Originally, photosynthesis models are applied assuming that the temperature responses of Rubisco kinetics are invariable across species (Niinemets & Tenhunen 1997; Bernacchi *et al*. 2001; Sharkey *et al*. 2007; Díaz-Espejo 2013) and, irrespective of the species considered, use the temperature functions provided for tobacco (Bernacchi *et al.* 2001; 2003; 2013) or for spinach (Jordan & Ogren 1984) to model photosynthesis in a range of environmental conditions. However, Rubisco kinetics varies among species and it also shown different patterns regarding the environmental conditions, principally by temperature (Zhu *et al.* 1998; Hikosaka *et al*. 2006; Archontoulis *et al.* 2012; Walker *et al*. 2013). Table 5 shows a bibliographic recompilation of the activation energy (ΔH_a) of Rubisco kinetic parameters, on different species grouped as C_3 plants from cool and warm habitats, intermediate C_3/C_4 plants and C_4 plants. The response of ΔH_a for the different Rubisco kinetics parameters showed differences among the groups of plants.

ΔH_a of k_{cat}^c showed the highest values in C_3 plants from warm habitats, with an average of 63.2 kJ mol^{-1}, while the lowest were found in the C_4 species with an average of 52.2 kJ mol^{-1} (Table 5). However, Sage (2002) reported a trend in increase ΔH_a in C_3 plants from cool habitats and C_4 plants compared to C_3 plants from warm habitats, which is not in agreement with the results of the present study. These discrepancies may be due to the difference in the number of plants species included in this study with regard to the reported by Sage (2002). These findings show that Rubisco of C_3 species from warm environments has evolved towards a more plastic response to temperature changes.

ΔH_a of K_c showed the highest and lowest values – 65.4 and 48.8 kJ mol^{-1} – in C_3 plants from warm and cool habitats, respectively (Table 5). However, ΔH_a of K_c did not show statistical differences among the groups, due to the heterogeneity among the data. However, the compilation highlights the existence of species–specific differences in K_c with regard the temperature, even in plants grouped according to the prevailing environmental conditions and photosynthetic mechanism. Overall, these results evidence the importance of use the appropriate K_c of each species in the application of the photosynthetic models, as has been recently stressed (Diaz-Espejo 2013; Walker *et al.* 2013).

ΔH_a of $S_{c/o}$ presented a similar behavior that in K_c. However, in this case the differences among the groups were not large, being the C_3 plants from warm and cool habitats again those that showed the highest and lowest values – 27.7 and 25.3 kJ mol-1 –, respectively (Table 5). This pattern in the activation energy of $S_{c/o}$ also was observed in this Thesis among the different species of *Flaveria* (to more detail see chapter 6), which demonstrates no difference in the thermal sensitivity of $S_{c/o}$ between C_3 and C_4 Rubiscos. Nevertheless, to confirm this affirmation the study of ΔH_a of $S_{c/o}$ should be extended to more species.

Due to the lack of information about the K_{RuBP} in literature, most of the data of ΔH_a of K_{RuBP} were obtained with the data reported in this Thesis (Chapter 6). ΔH_a of K_{RuBP} did not show statistical differences among the warm C_3, C_3/C_4 and C_4 plants, being these data from the *Flaveria* species reported in this study. However, the only data found in literature were in *Atriplex glabriuscula*, a C_3 plant from cool habitats that showed a lower value ΔH_a, being this 30% lower than the reported by us in the two C_3 *Flaveria pringlei* and *cronquistii* (Table 5). These findings suggest that C_3 plants from cool habitats have a lowest plastic response to temperature changes. Nevertheless, as in the case of the $S_{c/o}$, more measurements are necessary in different species to confirm this finding.

In conclusion, these differences in the activation energy of the Rubisco kinetic parameters among species, underscores the importance of improved understanding of variations in temperature responses of biochemical determinants of photosynthesis in models predicting carbon gain under future conditions.

Table 5. Temperature dependence parameters, scaling constant (C) and the activation energy (ΔH_a, kJ mol^{-1}) of the Rubisco maximum carboxylase turnover rate (k_{cat}^c), the Michaelis-Menten constant for CO_2 (K_c), the specificity factor ($S_{c/o}$) and the Michaelis-Menten constant for RuBP (K_{RuBP})

Specie	Reference	k_{cat}		K_c		$S_{c/o}$		K_{RuBP}	
		c	**ΔH_a**	**c**	**ΔH_a**	**c**	**ΔH_a**	**c**	**ΔH_a**
C_3 plants from cool habitats		**22.3**	**55.3**	**28.4**	**48.4**	**-2.4**	**-25.3**	**32.9**	**59.4**
Alopecurus alpinus	Tieszen *et al.* (1953)	25.2	62.5	-	-	-	-	-	-
Arctagrostis latifolia	Tieszen *et al.* (1953)	20.4	50.5	-	-	-	-	-	-
Atriplex glabriuscula	Badger & Collatz (1977)	28.0	69.3	31.8	56.3	-	-	32.9	59.4
Chenopodium album	Sage *et al*. (1995)	21.3	52.9	-	-	-	-	-	-
Dupontia fisheri	Tieszen *et al.* (1953)	22.5	55.8	-	-	-	-	-	-
Espeletia schultzii	Castrillo (1995)	18.2	45.1	26.2	43.1	-	-	-	-
Pinus sylvestris	Gezelius (1975)	23.2	57.3	-	-	-	-	-	-
Poa arctica	Sage (2002)	24.3	60.1	-	-	-	-	-	-
Poa pratensis	Sage (2002)	20.5	50.8	-	-	-	-	-	-
Solanum tuberosum	Sage (2002)	22.0	54.6	-	-	-	-	-	-
Spinacia oleracea	Badger & Andrews (1974)	18.5	46.2	-	-	-	-	-	-
Triticum aestivum	Makino *et al.* (1988)	23.4	58.1	-	-	-	-	-	-
Solanum commersonii	Huner *et al.* (1981)	-	-	26.7	42.8	-	-	-	-
Solanum tuberosum	Huner *et al.* (1981)	-	-	27.7	45.8	-	-	-	-

Spinacia oleracea	Jordan & Ogren (1984)	-	-	36.6	70.1	-2.8	-26.2	-	-
Triticum aestivum	(Mächler & Nösberger (1980)	-	-	21.5	32.4	-	-	-	-
Epilobium hirsutum	Ghashghaie & Cornic (1994)	-	-	-	-	-11.2	-47.3	-	-
Triticum aestivum	Hall & Keys (1983)	-	-	-	-	4.1	-9.0	-	-
Triticum aestivum	Haslam *et al.* (2005)	-	-	-	-	-1.8	-24.1	-	-
Spinacea oleracea	Uemura *et al.* (1997)	-	-	-	-	-2.7	-25.9	-	-
Spinacea oleracea	Zhu *et al.* (1998)	-	-	-	-	-0.1	-19.4	-	-
C_3 plants from warm habitats		**25.6**	**63.2**	**33.4**	**61.7**	**-3.3**	**-27.4**	**43.2**	**85.2**
Flaveria pringlei	This study	22.6	53.9	32.3	59.8	-3.0	-26.7	42.2	82.7
Flaveria cronquistii	This study	22.9	54.0	38.7	76.1	-2.5	-25.3	44.2	87.7
Agropyron smithii	Monson *et al.* (1982)	22.0	54.7	24.2	38.0	-	-	-	-
Arachis hypogaea	Sage (2002)	24.4	60.6	-	-	-	-	-	-
Astragalus flavus	Weber *et al.* (1977)	34.0	84.4	-	-	-	-	-	-
Astragalus rafaelensis	Weber *et al.* (1977)	31.8	78.8	-	-	-	-	-	-
Capsicum chinense	Sage (2002)	22.0	54.5	-	-	-	-	-	-
Flaveria pringlei	Sage (2002)	20.4	50.4	-	-	-	-	-	-
Glycine max	Bowes *et al*. (1975)	19.3	47.7	-	-	-	-	-	-
Gossypium hirsutum	Sage (2002)	24.0	59.4	-	-	-	-	-	-

Nicotiana tabacum	Crafts-Brandner & Salvucci (2000)	20.8	51.0	-	-	-	-	-	-
Oryza sativa	Makino *et al.* (1988)	24.3	60.3	-	-	-	-	-	-
Pueraria lobata	Sage (2002)	25.8	63.8	-	-	-	-	-	-
Solanum lycopersicum	Weber *et al.* (1977)	29.4	72.8	-	-	-	-	-	-
Stanleya pinnata	Weber *et al.* (1977)	31.5	78.1	-	-	-	-	-	-
Trifolium repens	Lehnherr *et al.* (1985)	34.7	86.1	37.3	70.8	-2.0	-23.7	-	-
Glycine max	Laing *et al*. (1974)	-	-	34.6	63.6	-	-	-	-
Beta maritima sp. marcosii	Galmés *et al.* (2005)	-	-	-	-	-3.2	-27.4	-	-
Diplotaxis ibicensis Pau	Galmés *et al.* (2005)	-	-	-	-	-4.2	-29.9	-	-
Hypericum balearicum	Galmés *et al.* (2005)	-	-	-	-	-3.8	-28.7	-	-
Limonium magallufianum	Galmés *et al.* (2005)	-	-	-	-	-3.8	-29.0	-	-
Lysimachia minoricensis	Galmés *et al.* (2005)	-	-	-	-	-2.7	-25.9	-	-
Pistacia lentiscus	Galmés *et al.* (2005)	-	-	-	-	-3.0	-26.9	-	-
Urtica atrovirens ssp. bianorii	Galmés *et al.* (2005)	-	-	-	-	-2.7	-25.9	-	-
Phaseolus vulgaris	Ghashghaie & Cornic	-	-	-	-	-5.7	-33.4	-	-

	(1994)								
Amaranthus hybridus	Jordan & Ogren (1984)	-	-	-	-	-5.7	-33.0	-	-
Flaveria pringlei	Zhu *et al.* (1998)	-	-	-	-	-0.6	-21.0	-	-
Intermediate C_3/C_4 plants		**21.9**	**51.7**	**34.6**	**65.4**	**-3.1**	**-26.8**	**44.4**	**88.3**
Flaveria angustifolia	This study	21.5	50.8	35.3	67.1	-3.2	-27.3	43.7	86.8
Flaveria floridana	This study	22.2	52.5	33.8	63.6	-2.9	-26.3	45.0	89.8
C_4 plants		**21.2**	**52.2**	**31.1**	**55.6**	**-3.2**	**-26.8**	**44.4**	**88.3**
Flaveria bidentis	This study	20.4	47.6	31.1	55.5	-2.8	-26.0	43.8	86.8
Flaveria trinervia	This study	21.6	50.8	31.0	55.6	-3.5	-27.5	44.9	89.7
Amaranthus retroflexus	Sage (2002)	24.3	60.4	-	-	-	-	-	-
&ropogon gerardi	Tieszen *et al.* (1953)	20.8	51.8	-	-	-	-	-	-
Bouteloua gracilis	Pittermann & Sage (2000)	20.8	51.8	-	-	-	-	-	-
Cynodon dactylon	Sage (2002)	21.1	52.3	-	-	-	-	-	-
Digitaria sanguinalis	Sage (2002)	18.8	46.6	-	-	-	-	-	-
Flaveria trinervia	Sage (2002)	20.3	50.4	-	-	-	-	-	-
Muhlenbergia montana	Sage (2002)	21.2	52.7	-	-	-	-	-	-
Portulaca oleracea	Sage (2002)	21.7	53.7	-	-	-	-	-	-
Sorghum bicolor	Tieszen *et al.* (1953)	23.5	58.1	-	-	-	-	-	-
Zoysia japonica	Sage (2002)	20.1	49.8	-	-	-	-	-	-

Chapter 8

CONCLUSIONS

From the above Results and Discussion, a series of conclusions can be drawn which respond to the objectives established in the present thesis.

1. *To identify the effect of high temperature and water deficit on the main physiological processes related to plant carbon balance in three of the world's most important crops.*

1. As a general trend, both stresses were detrimental in growth, gas exchange and biochemical parameters measured. However, some parameters only showed a negative effect when both stresses were acting at a time. This would indicate the importance of the additive effect of temperature rising and drought stresses.

2. Leaf carbon balance was highly affected by water deficit in the three species. However, the interaction of both stresses had the largest detrimental effect on leaf carbon balance and water use efficiency, being these two highly explicative of the changes in total plant biomass.

3. CO_2 assimilation was mainly affected by water deficit than by temperature in the three species. Under water deficit, the diffusive limitations were increased due to a decrease in stomatal and mesophyll conductances. However, the combination of both stresses in these parameters were not more detrimental that the individual effect of water deficit.

4. The acclimation of each of the three species to high temperature and water deficit was species-specific. However, rice and maize showed better acclimation to high temperature than wheat. This lower acclimation in wheat to high temperature suggests that the climate of domestication of each species plays an important role in its acclimation grade.

2. *To study the role of Rubisco on the plant response processes to high temperature and water deficit.*

5. The results obtained in this study demonstrate that the species-specific differences in Rubisco and PEPC kinetics are large enough to affect modelling of C_3 and C_4 photosynthesis. Therefore, these observations can improve the estimation of parameters obtained from photosynthetic models.

6. Biochemical limitations on photosynthesis were predominant under high temperature and the combination of high temperature and water deficit. This biochemical limitations were mainly attributed to decreases in Rubisco activation, which comes related with inhibition of Rubisco activase.

7. The Rubisco kinetic parameters showed a different thermal dependence among related species of *Flaveria* with different photosynthetic mechanisms – C_3, C_3/C_4 and C_4 –. These differences on thermal dependencies among photosynthetic mechanisms demonstrate that Rubisco kinetics no only respond to the thermal environment to which the species is adapted. Finally, the thermal dependencies in Rubisco kinetics from C_3, C_3/C_4 and C_4 species obtained in this study could be highly useful to fitting photosynthetic models.

REFERENCES LIST

Ainsworth E.A. & Ort D.R. (2010) How do we improve crop production in a warming world? *Plant Physiology* **154**, 526–30.

Alley R., Lynch-Stieglitz J. & Severinghaus J. (1999) Global Climate Change. *The National Academy of Sciences* **96**, 9987–9988.

Alscher R. & Cumming J. (1990) *Stress Responses in Plants: Adaptation and Acclimation Mechanisms*. Wiley-Liss, Inc, New York.

Anderson J., Badruzsaufari E., Schenk P., Manners J., Desmond O., Ehler C., *et al.* (2004) Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in. *The Plant Cell* **16**, 3460–3479.

Andrews T., Hudson G., Mate C., von Caemmerer S., Evans J. & Arvidsson Y. (1995) Rubisco – the consequences of altering its expression and activation in transgenic plants. *Journal of Experimental Botany* **46**, 1293–1300.

Athanasiou K., Dyson B.C., Webster R.E. & Johnson G.N. (2010) Dynamic acclimation of photosynthesis increases plant fitness in changing environments. *Plant Physiology* **152**, 366–373.

Atkin O.K., Scheurwater I. & Pons T. (2006) High thermal acclimation potential of both photosynthesis and respiration in two lowland Plantago species in contrast to an alpine congeneric. *Global Change Biology* **12**, 500–515.

Atkin O.K., Bruhn D., Hurry V.M. & Tjoelker M.G. (2005) The hot and the cold: unravelling the variable response of plant respiration to temperature. *Functional Plant Biology* **32**, 87–105.

Atkin O.K. & Tjoelker M.G. (2003) Thermal acclimation and the dynamic response of plant respiration to temperature. *Trends in Plant Science* **8**, 343–351.

Bernacchi C.J., Portis A.R., Nakano H., von Caemmerer S. & Long S.P. (2002) Temperature Response of Mesophyll Conductance. Implications for the Determination of Rubisco Enzyme Kinetics and for Limitations to Photosynthesis *in Vivo*. *Plant Physiology* **130**, 1992–1998.

Bernacchi C.J., Singsaas E.L., Pimentel C., Portis Jr A. R. & Long S.P. (2001) Improved temperature response functions for models of Rubisco-limited photosynthesis. *Plant, Cell and Environment* **24**, 253–259.

Berry J. & Björkman O. (1980) Photosynthetic response and adaptation to temperature in higher-plants. *Physiology and Plant Molecular Biology* **31**, 491–543.

Boese S.R. & Huner N.P. (1990) Effect of growth temperature and temperature shifts on spinach leaf morphology and photosynthesis. *Plant Physiology* **94**, 1830–1836.

Bota J., Medrano H. & Flexas J. (2004) Is photosynthesis limited by decreased Rubisco activity and RuBP content under progressive water stress? *New Phytologist* **162**, 671–681.

Boutraa T. (2010) Improvement of water use efficiency in irrigated agriculture: A review. *Journal of Agronomy* **9**, 1–8.

Bowler C. & Fluhr R. (2000) The role of calcium and activated oxygens as signals for controlling cross-tolerance. *Trends in Plant Science* **5**, 241–246.

Boyer J. (1982) Plant productivity and environment. *Science* **218**, 443–448.

Brooks A. & Farquhar G.D. (1985) Effect of temperature on the CO_2/O_2 specificity of ribulose- 1,5-bisphosphate carboxylase/oxygenase and the rate of respiration in the light. *Planta* **165**, 397–406.

von Caemmerer S. (2000) *Biochemical Models of Leaf Photosynthesis*. CSIRO, Collingwood.

von Caemmerer S. & Farquhar G. (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* **153**, 376–387.

Campbell C., Atkinson L., Zaragoza-Castells J., Lundmark M., Atkin O. & Hurry V. (2007) Acclimation of photosynthesis and respiration is asynchronous in response to changes in temperature regardless of plant functional group. *The New Phytologist* **176**, 375–89.

Campbell W.J. & Ogren W.L. (1990) Electron Transport through photosystem I Stimulates Light Activation of Ribulose Bisphosphate Carboxylase/Oxygenase (Rubisco) by Rubisco Activase. *Plant Physiology* **94**, 479–484.

Cardinale M., Casini M. & Arrhenius F. (2002) The influence of biotic and abiotic factors on the growth of sprat (Sprattus sprattus) in the Baltic Sea. *Aquatic Living Resourses* **15**, 273–281.

Casal J.J. (2002) Environmental cues affecting development. *Current Opinion in Plant Biology* **5**, 37–42.

Challinor A.J., Ewert F., Arnold S., Simelton E. & Fraser E. (2009) Crops and climate change: progress, trends, and challenges in simulating impacts and informing adaptation. *Journal of Experimental Botany* **60**, 2775–2789.

Chaves M., Pereira J., Maroco J., Rodrigues M., Ricardo C., Osório M., *et al.* (2002) How Plants Cope with Water Stress in the Field? Photosynthesis and Growth. *Annals of Botany* **89**, 907–916.

Chaves M.M., Flexas J. & Pinheiro C. (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Annals of Botany* **103**, 551–560.

Christensen J., Hewitson B., Busuioc A., Chen A., Gao X., Held I., *et al.* (2007) Regional climate projections. In: *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change.* (Eds S. Solomon, D. Qin, M, Manning, Z. Chen, M. Marquis, K. Averyt, et al.), pp. 847–940. Cambridge University Press, Cambridge, UK, and New York.

Cowling S. A. & Sage R.F. (1998) Interactive effects of low atmospheric CO_2 and elevated temperature on growth, photosynthesis and respiration in Phaseolus vulgaris. *Plant, Cell and Environment* **21**, 427–435.

Crafts-Brandner S.J. & Salvucci M.E. (2000) Rubisco activase constrains the photosynthetic potential of leaves at high temperature and CO_2. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 13430–13435.

Craufurd P. & Peacock J. (1993) Effect of heat and drought stress on sorghum. *Experimental Agriculture* **29**, 77–86.

DeWitt T. & Scheiner S. (2004) *Phenotypic Plasticity. Functional and Conceptual Approaches.* Oxford University Press., Oxford.

Diaz-Espejo A. (2013) New challenges in modelling photosynthesis: temperature dependencies of Rubisco kinetics. *Plant, Cell & Environment* **36**, 2104–2107.

Dore M.H.I. (2005) Climate change and changes in global precipitation patterns: what do we know? *Environment International* **31**, 1167–1181.

Dracup J. A., Lee K.S. & Paulson E.G. (1980) On the definition of droughts. *Water Resources Research* **16**, 297–302.

Easterling W., Aggarwal P., Batima P., Brander K., Erda L., Howden S., *et al.* (2007) Food, fibre and forest products. In: *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change.* (Eds S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K. Averyt, et al.), University Press, Cambridge., Cambridge.

Evans J. & von Caemmerer S. (2011) Enhancing photosynthesis. *Plant Physiology*.

FAO (2010) The state of food and Agriculture.

FAO (2009) The state of food and agriculture. *Rome: UAGST/FAO*.

Farquhar G.D., von Caemmerer S. & Berry J.A. (1980) A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. *Planta* **149**, 78–90.

Field C. (2001) Plant physiology of the "missing" carbon sink. *Plant Physiology* **125**, 25–28.

Field C., Behrenfeld M., Randerson J. & Falkowski P. (1998) Primary production of the biosphere: integrating terrestrial and oceanic components. *Science* **281**, 237–240.

Fischer G., Tubiello F.N., van Velthuizen H. & Wiberg D. A. (2007) Climate change impacts on irrigation water requirements: Effects of mitigation, 1990–2080. *Technological Forecasting and Social Change* **74**, 1083–1107.

Fitter A. & Hay R. (2002) *Environmental Physiology of Plants*. Academic Press, San Diego, CA.

Flexas J., Barbour M.M., Brendel O., Cabrera H.M., Carriquí M., Díaz-Espejo A., *et al.* (2012) Mesophyll diffusion conductance to CO_2: an unappreciated central player in photosynthesis. *Plant Science : an International Journal of Experimental Plant Biology* **193-194**, 70–84.

Flexas J., Bota J., Loreto F., Cornic G. & Sharkey T.D. (2004) Diffusive and metabolic limitations to photosynthesis under drought and salinity in C_3 plants. *Plant Biology (Stuttgart, Germany)* **6**, 269–279.

Flexas J., Galmés J., Ribas-Carbó M. & Medrano H. (2005) The effects of water stress on plant respiration. In: *Advances in Photosynthesis and Respiration. Plant Respiration: From Cell to Ecosystem*. (Eds H. Lambers & M. Ribas-Carbó), pp. 85–94. Kluwer Academic Publishers, Dordrecht.

Flexas J. & Medrano H. (2002a) Drought- inhibition of photosynthesis in C_3 plants: stomatal and non stomatal limitations revisited. *Annals of Botany* **89**, 183–189.

Flexas J. & Medrano H. (2002b) Photosynthetic responses of C_3 plants to drought. In: *Advances in Plant Physiology*, IV. (Ed. A. Hemantaranjan), Jodhpur: Scientific Publishers.

Flexas J., Ribas-Carbó M., Bota J., Galmés J., Henkle M., Martínez-Cañellas S., *et al.* (2006) Decreased Rubisco activity during water stress is not induced by decreased relative water content but related to conditions of low stomatal conductance and chloroplast CO_2 concentration. *The New Phytologist* **172**, 73–82.

Flexas J., Ribas-Carbó M., Diaz-Espejo A., Galmés J. & Medrano H. (2008) Mesophyll conductance to CO_2: current knowledge and future prospects. *Plant, Cell & Environment* **31**, 602–621.

Friend A. & Woodward F. (1990) Evolution and ecophysiological responses of mountain plants to the growing season environment. *Advances in Ecological Research* **20**, 59–124.

Galmés J., Aranjuelo I., Medrano H. & Flexas J. (2013) Variation in Rubisco content and activity under variable climatic factors. *Photosynthesis Research* **117**, 73–90.

Galmés J., Cifre J., Medrano H. & Flexas J. (2005) Modulation of relative growth rate and its components by water stress in Mediterranean species with different growth forms. *Oecologia* **145**, 21–31.

Galmés J., Conesa M.À., Ochogavía J.M., Perdomo J.A., Francis D.M., Ribas-Carbó M., *et al.* (2011a) Physiological and morphological adaptations in relation to water use efficiency in Mediterranean accessions of Solanum lycopersicum. *Plant, Cell & Environment* **34**, 245–260.

Galmés J., Flexas J., Medrano H., Niinemets Ü. & Valladares F. (2012) Ecophysiology of photosynthesis in semi-arid environments. In: *Terrestrial Photosynthesis in a Changing Environment*. (Eds J. Flexas, F. Loreto & H. Medrano), pp. 453–469. Cambridge University Press, Cambridge.

Galmés J., Kapralov M. V, Andralojc P.J., Conesa M.À., Keys A.J., Parry M. A. J., *et al.* (2014) Expanding knowledge of the Rubisco kinetics variability in plant species: environmental and evolutionary trends. *Plant, Cell & Environment* **37**, 1989–2001.

Galmés J., Medrano H. & Flexas J. (2007a) Photosynthesis and photoinhibition in response to drought in a pubescent (var. minor) and a glabrous (var. palaui) variety of Digitalis minor. *Environmental and Experimental Botany* **60**, 105–111.

Galmés J., Medrano H. & Flexas J. (2007b) Photosynthetic limitations in response to water stress and recovery in Mediterranean plants with different growth forms. *The New Phytologist* **175**, 81–93.

Galmés J., Ribas-Carbó M., Medrano H. & Flexas J. (2007c) Response of leaf respiration to water stress in Mediterranean species with different growth forms. *Journal of Arid Environments* **68**, 206–222.

Galmés J., Ribas-Carbó M., Medrano H. & Flexas J. (2011b) Rubisco activity in Mediterranean species is regulated by the chloroplastic CO_2 concentration under water stress. *Journal of Experimental Botany* **62**, 653–665.

Gao F., Qian M. & Miao X. (2000) Mechanical analysis of the immediate roof subjected to given deformation of the main roof. *Chinese Journal of Rock Mechanics and Engineering* **19**, 145–148.

Gornall J., Betts R., Burke E., Clark R., Camp J., Willett K., *et al.* (2010) Implications of climate change for agricultural productivity in the early twenty-first century. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **365**, 2973–2989.

Grassi G. & Magnani F. (2005) Stomatal, mesophyll conductance and biochemical limitations to photosynthesis as affected by drought and leaf ontogeny in ash and oak trees. *Plant, Cell and Environment* **28**, 834–849.

Greer D.H. & Weedon M.M. (2012) Modelling photosynthetic responses to temperature of grapevine (Vitis vinifera cv. Semillon) leaves on vines grown in a hot climate. *Plant, Cell & Environment* **35**, 1050–1064.

Griggs D.J. (2001) C LIMATE C HANGE 2001: THE SCIENTIFIC BASIS. *Cambridge University* **57**, 267–269.

Gunderson C., Norby R. & Wullschleger S. (2000) Acclimation of photosynthesis and respiration to simulated climatic warming in northern and southern populations of Acer saccharum: laboratory and field evidence. *Tree Physiology* **20**, 87–96.

Gutteridge S. & Keys A.J. (1985) The significance of Ribulose-1,5-bisphosphate carboxylase in determining the effects of the environment on photosynthesis and photorespiration. In: *Photosynthetic Mechanisms and the Environment*. (Eds J. Barber & N.R. Baker), pp. 259–285. Elsevier Science, Amsterdam.

Hall N.P. & Keys A.J. (1983) Temperature dependence of the enzymic carboxylation and oxygenation of ribulose 1,5-bisphosphate in relation to effects of temperature on photosynthesis. *Plant physiology* **72**, 945–948.

Hansen J., Ruedy R., Sato M. & Lo K. (2010) GLOBAL SURFACE TEMPERATURE CHANGE. *Reviews of Geophysics* **48**, 1–29.

Hendrickson L., Ball M.C., Wood J.T., Chow W.S. & Furbank R.T. (2004) Low temperature effects on photosynthesis and growth of grapevine. *Plant, Cell and Environment* **27**, 795–809.

Heyne E. & Brunson A. (1940) Genetic studies of heat and drought tolerance in maize. *American Society of Agronomy* **32**, 803–814.

Hozain M.I., Salvucci M.E., Fokar M. & Holaday A.S. (2010) The differential response of photosynthesis to high temperature for a boreal and temperate Populus species relates to differences in Rubisco activation and Rubisco activase properties. *Tree Physiology* **30**, 32–44.

Hussain M., Malik M. A., Farooq M., Ashraf M.Y. & Cheema M. A. (2008) Improving Drought Tolerance by Exogenous Application of Glycinebetaine and Salicylic Acid in Sunflower. *Journal of Agronomy and Crop Science* **194**, 193–199.

IPCC (2013) *Working Group I Contribution to the IPCC Fifth Assessment Report Climate Change 2013: The Physical Science Basis Summary for Policymakers*.

Jiang Y. & Huang B. (2001) Drought and heat stress injury to two cool season turfgrasses in relation to antioxidant metabolism and lipid peroxidation. *Crop Science* **41**, 436–442.

Jordan D.B. & Ogren W.L. (1984) The CO_2/O_2 specificity of ribulose 1,5-bisphosphate carboxylase/oxygenase. *Planta* **161**, 308–313.

Kemp P. & Williams G.I. (1980) A physiological basis for niche separation between Agropyron smithii C_3 and Bouteloua gracilis C_4. *Ecology* **61**, 846–858.

Kobza J. & Edwards G.E. (1987) Influences of leaf temperature on photosynthetic carbon metabolism in wheat. *Plant Physiology* **83**, 69–74.

Kubien D.S., Whitney S.M., Moore P. V & Jesson L.K. (2008) The biochemistry of Rubisco in Flaveria. *Journal of Experimental Botany* **59**, 1767–1777.

Laing W., Ogren W. & Hageman R. (1974) Regulation of soybean net photosynthetic CO_2 fixation by the interaction of CO_2, O_2, and ribulose 1, 5-diphosphate carboxylase. *Plant Physiology* **54**, 678–685.

Lambers H., Chapin F. & Pons T. (1998) *Plant Physiological Ecology*. Springer, New York.

Lambers H. & Ribas-Carbó M. (2005) *Advances in Photosynthesis and Respiration. Plant Respiration: From Cell to Ecosystem.* Urbana.

Law R. & Crafts-Brandner S. (1999) Inhibition and acclimation of photosynthesis to heat stress is closely correlated with activation of ribulose-1,5-bisphosphate Carboxylase/Oxygenase. *Plant Physiology* **120**, 173–182.

Lawlor D. & Mitchell R. (2000) Wheat. In: *Climate Change and Global Crop Productivity.* (Eds K. Reddy & H. Hodges), CAB International, Wallingford, UK.

Lawlor D.W. & Tezara W. (2009) Causes of decreased photosynthetic rate and metabolic capacity in water-deficient leaf cells: a critical evaluation of mechanisms and integration of processes. *Annals of Botany* **103**, 561–579.

Lobell D. & Asner G. (2003) Climate and management con- tributions to recent trends in U.S. agricultural yields. *Science* **299**, 1032.

Long S.P. & Ort D.R. (2010) More than taking the heat: crops and global change. *Current Opinion in Plant Biology* **13**, 241–248.

Van de Loo F.J. & Salvucci M.E. (1996) Activation of ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) involves Rubisco activase Trp16. *Biochemistry* **35**, 8143–8148.

Loveys B.R., Scheurwater I., Pons T.L., Fitter a. H. & Atkin O.K. (2002) Growth temperature influences the underlying components of relative growth rate: an investigation using inherently fast- and slow-growing plant species. *Plant, Cell and Environment* **25**, 975–988.

Ludlow M. (1989) *Strategies of Response to Water Stress. in Structural and Functional Responses to Environmental Stresses.* (Eds K.. Kreeb, H. Ritcher & T.. Hinckley), SPB Academic Publishing, The Hague.

Luomala E., Laitinen K., Sutinen S., Kellomaki S. & Vapaavuori E. (2005) Stomatal density, anatomy and nutrient concentrations of Scots pine needles are affected by elevated CO_2 and temperature. *Plant, Cell & Environment* **28**, 733–749.

Machado S. & Paulsen G.M. (2001) Combined effects of drought and high temperature on water relations of wheat and sorghum. *Plant and Soil* **233**, 179–187.

Makino A., Nakano H. & Mae T. (1994) Effects of Growth Temperature on the Responses of Ribulose-1,5-Bisphosphate Carboxylase, Electron Transport Components , and Sucrose Synthesis Enzymes to Leaf Nitrogen in Rice , and Their Relationships to Photosynthesis. *Plant Physiology* **105**, 1231–1238.

McKersie B., Bowley S. & Jones K. (1999) Winter survival of transgenic alfalfa overexpressing superoxide dismutase. *Plant Physiology* **119**, 839–848.

Medrano H., Flexas J. & Galmés J. (2009) Variability in water use efficiency at the leaf level among Mediterranean plants with different growth forms. *Plant and Soil* **317**, 17–29.

Mittler R. (2006) Abiotic stress, the field environment and stress combination. *Trends in Plant Science* **11**, 15–19.

Mittler R., Vanderauwera S., Gollery M. & Van Breusegem F. (2004) Reactive oxygen gene network of plants. *Trends in Plant Science* **9**, 490–498.

Moffat A. (2002) Plant genetics. Finding new ways to protect drought- stricken plants. *Science* **296**, 1226–1229.

Monson R.K., Stidham M.A., Iii G.J.W., Edwards G.E. & Uribe E.G. (1982) Temperature Dependence of Photosynthesis in Agropyron smithii Rydb .'. 921–928.

Nagai T. & Makino A. (2009) Differences between rice and wheat in temperature responses of photosynthesis and plant growth. *Plant & Cell Physiology* **50**, 744–755.

National Climatic Data center

National Research Council (2010) *Advancing the Science of Climate Change*. The National Academies Press, Washington.

Nelson G., Mark W R., Koo J., Robertson R., Sulser T., Zhu T., *et al.* (2009) *Climate Change: Impact on Agriculture and Costs of Adaptation*.

Nguyen N. (2008) Global climate changes and rice food security. *FAO*, 24–30.

Ogren W. (1984) Photorespiration: pathways, regulation, and modification. *Annual Review of Plant Physiology* **35**, 415–442.

Parry M. A. J., Andralojc P., Khan S., Lea P. & Keys A. (2002) Rubisco Activity: Effects of Drought Stress. *Annals of Botany* **89**, 833–839.

Perdomo P., Murphy J. & Berkowitz G. (1996) Physiological changes associated with performance of Kentucky bluegrass cultivars during summer stress. *Hort Science* **31**, 1182–1186.

Pinheiro C. & Chaves M.M. (2011) Photosynthesis and drought: can we make metabolic connections from available data? *Journal of Experimental Botany* **62**, 869–882.

Poorter H. & Nagel O. (2000) The role of biomass allocation in the growth response of plants to different levels of light, CO2, nutrients and water: a quantitative review. *Functional Plant Biology* **27**, 595–607.

Poorter H., Niinemets Ü., Poorter L., Wright I.J. & Villar R. (2009) Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. *New Phytologist* **182**, 565–588.

Porter J.R. & Semenov M. a (2005) Crop responses to climatic variation. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **360**, 2021–2035.

Portis A.R. (2003) Rubisco activase - Rubisco's catalytic chaperone. *Photosynthesis Research* **75**, 11–27.

Potter J. & Jones J. (1977) No TitleLeaf area partitioning as an important factor in growth. *Plant Physiology* **59**, 10–14.

Quinn P. & Williams W. (1985) Environmentally induced changes in chloroplast membranes and their effects on photo- synthetic function. In: *Photosynthetic Mechanisms and the Environment.* (Eds J. Barber & N. Baker), pp. 1–47. Elsevier Science Publishers, Amsterdam.

Reddy A.R., Rasineni G.K. & Raghavendra A.S. (2010) The impact of global elevated CO_2 concentration on photosynthesis and plant productivity. *Current Science* **99**, 46–57.

Reynolds M.P. & Rodomiro O. (2010) Adapting Crops to Climate Change: a Summary. In: *Climate Change and Crop Production*. (Ed. M.P. Reynolds), pp. 1–8. Cabi, Wallingford.

Rizhsky L., Liang H. & Mittler R. (2002) The combined effect of drought stress and heat shock on gene expression in tobacco. *Plant Physiology* **130**, 1143–1151.

Rizhsky L., Liang H., Shuman J., Shulaev V., Davletova S. & Mittler R. (2004) When Defense Pathways Collide. The Response of Arabidopsis to a Combination of Drought and Heat Stress. *Plant Physiology* **134**, 1683–1696.

Rosenzweig C., Tubiello F., Goldberg R., Mills E. & Bloomfield J. (2002) Increased crop damage in the US from excess precipitation under climate change. *Global Environmental Change* **12**, 197–202.

Rucker K., Kvien C., Holbrook C. & Hook J. (1995) Identification of peanut genotypes with improved drought avoidance traits. *Peanut Science* **24**, 14–18.

Rustad L., Campbell J., Marion G., Norby R., Mitchell M., Hartley A., *et al.* (2001) A meta-analysis of the response of soil respiration, net nitrogen mineralization, and aboveground plant growth to experimental ecosystem warming. *Oecologia* **126**, 543–562.

Ruuska S., Andrews T., Badger M., Price G. & von Caemmerer S. (2000) The role of chloroplast electron transport and metabolites in modulating Rubisco activity in tobacco. Insights from transgenic plants with reduced amounts of cytochrome b/f

complex or glyceraldehyde 3-phosphate dehydrogenase. *Plant physiology* **122**, 491–504.

Sage R. & Pearcy R. (1987) The nitrogen use efficiency of C_3 and C_4 plants II. Leaf nitrogen effects on the gas exchange characteristics of Chenopodium album L. and Amaranthus retroflexus L. *Plant Physiology* **84**, 959–963.

Sage R.F. (2002) Variation in the kcat of Rubisco in C_3 and C_4 plants and some implications for photosynthetic performance at high and low temperature. *Journal of Experimental Botany* **53**, 609–620.

Sage R.F. & Kubien D.S. (2007) The temperature response of C_3 and C_4 photosynthesis. *Plant, Cell & Environment* **30**, 1086–1106.

Sage R.F. & Sharkey T.D. (1987) The Effect of Temperature on the Occurrence of O_2 and CO_2 Insensitive Photosynthesis in Field Grown Plants. *Plant Physiology* **84**, 658–664.

Sage R.F., Way D. a & Kubien D.S. (2008) Rubisco, Rubisco activase, and global climate change. *Journal of Experimental Botany* **59**, 1581–1595.

Salvucci M.E. & Crafts-brandner S.J. (2004) Relationship between the Heat Tolerance of Photosynthesis and the Thermal Stability of Rubisco Activase in Plants from Contrasting Thermal Environments. *Plant Physiology* **134**, 1460–1470.

Santrucek J. & Sage R. (1996) Acclimation of Stomatal Conductance to a CO_2 -enriched Atmosphere and Elevated Temperature in Chenopodium album. *Australian Journal of Plant Physiology* **23**, 467–478.

Savage D. & Jacobson L. (1935) The killing effect of heat and drought on buffalo grass and blue grama grass at Hays, Kansas. *American Society of Agronomy* **27**, 566–582.

Scafaro A.P., Von Caemmerer S., Evans J.R. & Atwell B.J. (2011) Temperature response of mesophyll conductance in cultivated and wild Oryza species with contrasting mesophyll cell wall thickness. *Plant, Cell & Environment* **34**, 1999–2008.

Scafaro A.P., Yamori W., Carmo-Silva a E., Salvucci M.E., von Caemmerer S. & Atwell B.J. (2012) Rubisco activity is associated with photosynthetic thermotolerance in a wild rice (Oryza meridionalis). *Physiologia Plantarum* **146**, 99–109.

Schär C., Vidale P.L., Lüthi D., Frei C., Häberli C., Liniger M.A., *et al.* (2004) The role of increasing temperature variability in European summer heatwaves. *Nature* **427**, 332–336.

Schrader S.M., Wise R.R., Wacholtz W.F., Ort D.R. & Sharkey T.D. (2004) Thylakoid membrane responses to moderately high leaf temperature in Pima cotton. *Plant, Cell and Environment* **27**, 725–735.

Schulze E., Beck E. & Müller-Hohenstein K. (2002) *Plant Ecology*. Spektrum Akademischer Verlag GmbH, Heidelberg.

Sharkey T.D. (2005) Effects of moderate heat stress on photosynthesis: importance of thylakoid reactions, rubisco deactivation, reactive oxygen species, and thermotolerance provided by isoprene. *Plant, Cell and Environment* **28**, 269–277.

Sharkey T.D. (1989) Evaluating the Role of Rubisco Regulation in Photosynthesis of C_3 Plants. *Philosophical Transactions of the Royal Society B: Biological Sciences* **323**, 435–448.

Sharkey T.D. & Seemann J.R. (1989) Mild water stress effects on carbon-reduction-cycle intermediates, ribulose bisphosphate carboxylase activity, and spatial homogeneity of photosynthesis in intact leaves. *Plant physiology* **89**, 1060–1065.

Sheffield J., Wood E.F. & Roderick M.L. (2012) Little change in global drought over the past 60 years. *Nature* **491**, 435–438.

Smil V. (2005) Do we need higher farm yields during the first half of the 21st century?. In: *Yields of Farmed Species*. (Eds R. Sylvester-Bradley & J. Wiseman), pp. 1–14. Nottingham University Press, Nottingham, UK.

Spreitzer R.J. & Salvucci M.E. (2002) Rubisco: structure, regulatory interactions, and possibilities for a better enzyme. *Annual Review of Plant Biology* **53**, 449–475.

Tcherkez G.G.B., Farquhar G.D. & Andrews T.J. (2006) Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 7246–7251.

Tenhunen J., Lange O., Gebel J., Beyschlag W. & Weber J. (1984) Changes in photosynthetic capacity, carboxylation efficiency and CO_2 compensation point associated with midday stomatal closure and midday depression of net CO_2 exchange in leaves of Quercus suber. *Planta* **193**, 193–203.

Tezara W., Mitchell V.J., Driscoll S.D. & Lawlor D.W. (1999) Water stress inhibits plant photosynthesis by decreasing coupling factor and ATP. *Letters to Nature* **401**, 914–917.

Turral H., Burke J. & Faurès J. (2011) *Climate Change, Water and Food Security*. Food and Agriculture Organization of the United Nations (FAO), Rome.

Valladares F., Gianoli E. & Gómez J.M. (2007) Ecological limits to plant phenotypic plasticity. *The New Phytologist* **176**, 749–763.

Vile D., Pervent M., Belluau M., Vasseur F., Bresson J., Muller B., *et al.* (2012) Arabidopsis growth under prolonged high temperature and water deficit: independent or interactive effects? *Plant, Cell & Environment* **35**, 702–718.

Walker B., Ariza L.S., Kaines S., Badger M.R. & Cousins A.B. (2013) Temperature response of in vivo Rubisco kinetics and mesophyll conductance in Arabidopsis thaliana: comparisons to Nicotiana tabacum. *Plant, Cell & Environment* **36**, 2108–2119.

Walters R.G. (2005) Towards an understanding of photosynthetic acclimation. *Journal of Experimental Botany* **56**, 435–447.

Warren C.R. (2008) Soil water deficits decrease the internal conductance to CO_2 transfer but atmospheric water deficits do not. *Journal of Experimental Botany* **59**, 327–334.

Warren C.R. & Dreyer E. (2006) Temperature response of photosynthesis and internal conductance to CO_2: results from two independent approaches. *Journal of Experimental Botany* **57**, 3057–3067.

Weis E. (1981) The temperature sensitivity of dark-inactivation and light-activation of the ribulose-1,5-bisphosphate carboxylase in spinach chloroplasts. *FEBS Letters* **129**, 197–200.

Whitney S.M., Houtz R.L. & Alonso H. (2011) Advancing our understanding and capacity to engineer nature's CO2-sequestering enzyme, Rubisco. *Plant Physiology* **155**, 27–35.

Woodward F. (1979) The differential temperature responses of the growth of certain plant species from different altitudes. II. Analyses of the control and morphology of leaf extension and specific leaf area of Phleum bertolonii D.C. and P. alpinum L. *New phytologist* **82**, 397–405.

Wright I.J., Reich P.B., Atkin O.K., Lusk C.H., Tjoelker M.G. & Westoby M. (2006) Irradiance, temperature and rainfall influence leaf dark respiration in woody plants: evidence from comparisons across 20 sites. *The New phytologist* **169**, 309–319.

Xiong L. & Yang Y. (2003) Disease Resistance and Abiotic Stress Tolerance in Rice Are Inversely Modulated by an Abscisic Acid–Inducible Mitogen-Activated Protein Kinase. *The plant cell* **15**, 745–759.

Xu Q. & Huang B. (2001) Morphological and physiological characteristics associated with heat tolerance in creeping bentgrass. *Crop Science* **41**, 127–133.

Yamori W. & von Caemmerer S. (2009) Effect of Rubisco activase deficiency on the temperature response of CO_2 assimilation rate and Rubisco activation state: insights from transgenic tobacco with reduced amounts of Rubisco activase. *Plant Physiology* **151**, 2073–2082.

Yamori W., Masumoto C., Fukayama H. & Makino A. (2012) Rubisco activase is a key regulator of non-steady-state photosynthesis at any leaf temperature and, to a lesser extent, of steady-state photosynthesis at high temperature. *The Plant Journal : for Cell and Molecular Biology* **71**, 871–880.

Yamori W., Suzuki K., Noguchi K., Nakai M. & Terashima I. (2006) Effects of Rubisco kinetics and Rubisco activation state on the temperature dependence of the photosynthetic rate in spinach leaves from contrasting growth temperatures. *Plant, Cell and Environment* **29**, 1659–1670.

Yamori W., Takahashi S., Makino A., Price G.D., Badger M.R. & von Caemmerer S. (2011) The roles of ATP synthase and the cytochrome b6/f complexes in limiting chloroplast electron transport and determining photosynthetic capacity. *Plant Physiology* **155**, 956–962.

Zhang M., Duan L., Tian X., He Z., Li J., Wang B., *et al.* (2007) Uniconazole-induced tolerance of soybean to water deficit stress in relation to changes in photosynthesis, hormones and antioxidant system. *Journal of Plant Physiology* **164**, 709–717.

Zhang R., Cruz J. A., Kramer D.M., Magallanes-Lundback M.E., Dellapenna D. & Sharkey T.D. (2009) Moderate heat stress reduces the pH component of the transthylakoid proton motive force in light-adapted, intact tobacco leaves. *Plant, Cell & Environment* **32**, 1538–1547.

Zhou Y., Lam H.M. & Zhang J. (2007) Inhibition of photosynthesis and energy dissipation induced by water and high light stresses in rice. *Journal of Experimental Botany* **58**, 1207–1217.